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THE BIOCHEMICAL JOURNAL

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I. ON THE PRESENCE OF CYANATE IN THE BLOOD.

By ERIK GOTTLIEB.

From the Physiological Laboratory, University of Copenhagen.

(*Received November 7th, 1925.*)

It has recently been asserted that considerable quantities of cyanate are found in the blood, namely 0·8–1·0 mg. HOCN per 100 cc. blood of the rabbit and cat [Montgomery, 1925].

These findings are of very great interest, since cyanates and cyanic acid, on account of their chemical nature, might be considered as precursors of urea in the liver [Bollman, Mann and Magath, 1924] as well as of ammonia in the kidneys [Nash and Benedict, 1921]. It might be presumed that, in neutral or in alkaline reaction, 2 mols. HOCN as well as 1 mol. $NH_4.OCN$ could give rise to urea, whilst cyanic acid, in acid reaction, is hydrolysed into ammonia and carbonic acid [cf. Werner, 1923]. Nowadays, such findings arouse more than ordinary interest, since, in the course of the last years, the conclusion has been reached that blood does not contain such quantities of ammonia and carbamate as were previously assumed[1], and also since the theory that cyanic acid gives rise to urea is considered by some to be one of the most probable [cf. Werner, 1923, and Montgomery, 1925].

However, in studying the ammonia formation in blood, I came to the conclusion that it was the same whether the blood, after collection, had stood either at acid or slightly alkaline reaction, so that it appeared unlikely that blood could contain such quantities of cyanate as recorded by Montgomery; for one would expect that the ammonia formation produced at acid reaction would be appreciably greater than that produced at alkaline reaction.

I therefore tried at first to determine the velocity of decomposition of cyanic acid. 1 cc. *N*/5 KOCN was mixed with 99 cc. "buffer mixture" of known reaction, and samples of 10 cc. were taken from time to time in order to determine the amount of ammonia formed, Folin's method being employed for this determination.

[1] Concerning the ammonium concentration in the blood, cf. Barnett [1917], Nash and Benedict [1921], Henriques and Gottlieb [1924], Parnas and Heller [1924], Parnas [1925]. According to Fauerholt [1924], the carbamate concentration in the blood must be very much smaller than the ammonium concentration. If, therefore, the ammonium-N concentration, according to the investigators mentioned above, is estimated at 0·03 mg. per 100 cc. blood at most, the carbamate-N concentration in a bicarbonate solution like blood can, according to Fauerholt, only be 0·2 % of this, or 0·00006 mg. per 100 cc. of blood.

Exp. 1. At 18° there were found the following values of NH_3-N, expressed in per cent. of the theoretically possible value formed at different reactions.

p_H	2·0	5·0	7·3	9·0
After 1 hour	98	78	1	1
,, 27 hours	100	99	3	2
,, 74 ,,	—	—	12	2
,, 17 days	—	—	91	7

Exp. 2. At p_H 2·0 the following NH_3-N values were found—in per cent. of the theoretically possible amount—formed at different temperatures.

	0°		18°
After 1 minute	6	After 1 minute	14
,, 5 minutes	9	,, 5 minutes	80
,, 10 ,,	21	,, 25 ,,	100
,, 30 ,,	34	,, 62 ,,	100

Exps. 1 and 2 show the dependence of the formation of ammonia on the p_H and temperature indicating that practically all the cyanate in a sufficiently acid reaction (p_H 2·0) is decomposed into ammonia in the course of less than half an hour at room temperature and in the dilution employed, namely $N/500$.

Afterwards an investigation was made as to the possibility of detecting the presence of a certain amount of cyanate added to blood by allowing samples of such blood to stand at acid and slightly alkaline reactions (p_H 2·0 and p_H 9·0 respectively), the difference in the amount of ammonia formed in acid and alkaline reactions being used as a standard for the amount of cyanate; for, from the experiments described above, we may conclude that all the cyanate present may be considered as being hydrolysed by standing 1 hour at p_H 2·0, whereas the amount of cyanate hydrolysed at p_H 9·0 is so small that it need not be taken into account. After the blood had stood for a certain time at acid and alkaline reactions, the amount of ammonia formed was determined by Folin's method, the samples being cooled at 0° previous to the addition of excess of potassium carbonate, and, subsequently, the ammonia formed was removed by aerating for 3 hours at a low temperature (0°–4°). The ammonia determinations themselves were carried out by titration with $N/280$ NaOH in a recipient of 1 cc. $N/280$ H_2SO_4 [Henriques and Gottlieb, 1924].

Four such experiments were carried out, namely two with blood and two with plasma. In the experiments with plasma 90–95 % of the amount of cyanate added could be estimated, whereas in the experiments with blood only 60–70 % could be recovered owing to the technical difficulties occasioned by the great quantity of haemoglobin in the blood.

Simultaneously with each of these experiments control experiments were carried out both with samples of normal plasma and with samples of blood, though without an addition of cyanate, the procedure in the controls being exactly the same; however, as it was impossible to detect any difference between the ammonia formation at acid reaction and that at alkaline reaction, it appears quite improbable that the small quantities of ammonia found were due to decomposition of cyanate.

One of these experiments described in detail may serve as an example. About 200 cc. blood collected from a dog were cooled rapidly, and mixed with pure NH_3-free Na oxalate. After 5 minutes' centrifugation the plasma was poured off and divided into two portions, one of which may be termed "P_O"; the other portion of 50 cc. was mixed with 0·5 cc. of a freshly prepared solution of KOCN (0·220 g. in 100 cc.). This mixture, which may be termed "P_{NCO}," should then contain 1·13 mg. of NCO′ per 100 cc.

From each of these two portions of plasma were then taken 4 samples of 5 cc. each, two of which were allowed to stand with 10 cc. of *N* tartaric acid, whilst the two others stood with a mixture of 10 cc. of *N* tartaric acid + 3 cc. of 3·5 *N* K_2CO_3, whereby the reaction of the mixture became approximately p_H 2·0 and p_H 9·0, respectively. After standing for two hours in a water-bath at 18°, the mixtures were cooled to 0°, and so much 3·5 *N* K_2CO_3 solution was added to them that each sample finally contained 10 cc. of 3·5 *N* K_2CO_3 altogether. (This was done on account of the possible amount of ammonia contained in the reagents employed, so that the blank value might be considered as being constant.) Subsequently the amount of ammonia contained in the eight samples was determined as described above.

(1)	5 cc. of P_O	on standing at	p_H 2·0	gave	0·005	mg.	NH_3-N
(2)	,,	,,	,,	,,	0·005	,,	
(3)	,,	,,	p_H 9·0	,,	0·005	,,	
(4)	,,	,,	,,	,,	0·004	,,	
(5)	5 cc. P_{NCO}	,,	p_H 2·0	,,	0·022	,,	
(6)	,,	,,	,,	,,	0·022	,,	
(7)	,,	,,	p_H 9·0	,,	0·005	,,	
(8)	,,	,,	,,	,,	0·006	,,	

As is seen from the above experiment, there is no essential difference between the values of NH_3-N found in normal plasma which had stood at acid and alkaline reactions respectively, and those found in cyanate plasma which had stood at alkaline reaction, the accuracy of the NH_3-N determination being about ±0·001 mg. Hence the quantities of NH_3 formed in these experiments cannot be due to the content of cyanate. In the samples (Nos. 5 and 6), however, which contained an addition of cyanate, and which had stood at acid reaction, an additional formation of 0·022 − 0·005 = 0·017 mg. NH_3-N was found, which corresponds to 1·02 mg. of NCO′ per 100 cc., or 90 % of the amount calculated.

Since the accuracy of the NH_3-N determination per 100 cc. of plasma is ± 0·001 mg., the possible error per 100 cc. is 0·04 mg. N, which corresponds to 0·12 mg. NCO′. This value is one-tenth of the values which Montgomery states that he has found in normal plasma.

In order to verify whether an organism is able to transform cyanate into urea, and since the literature contains nothing that throws light on the effect and the destiny of cyanate in the organism, experiments were subsequently carried out on three rabbits to which a cyanate solution was given

per os; these animals died however in the course of less than an hour, presenting characteristic symptoms of poisoning. The doses applied varied between 0·7 and 0·19 g. of NCO′ per kg. rabbit. The KOCN solution employed was very nearly neutralised and gave no positive reaction for cyanide.

Rabbits, however, occupy a somewhat special position in regard to nitrogen metabolism, for, unlike human beings and dogs, they are unable to excrete an excess of inorganic acids in the form of ammonium salts [cf. Pohl and Münzer, 1900; Kettner, 1902; Lyman and Raymond, 1919; and Palladin, 1924], and consequently the following experiment was carried out.

A dog weighing 16 kg. received a continuous intravenous injection of a *N*/3 cyanate solution at the rate of 0·42 g. cyanate-N per hour. This rate of N-transmission corresponds only to the metabolism of about 300 g. of flesh per 24 hours. However, the animal rapidly exhibited symptoms of poisoning similar to those observed in the experiments with the rabbits and died after 66 minutes. The animal had then received altogether 1·38 g. of NCO′, or 0·086 g. per kg.

During the experiment the amount of cyanate contained in the blood was determined by a method similar to that described above. At the beginning of the injection the value of cyanate found was 0·0 g. per 100 cc. of plasma; after 60 minutes' injection the value was 14·4 mg., and, after death, 15·1 mg.

It is possible that a certain amount of the injected cyanate may have been excreted, or transformed into other substances, but the figures obtained indicate that there has been no time for a significant excretion to take place. For, if one assumes a uniform distribution of the salt in the quantity of water contained in the organism, and estimates this to be two-thirds of the weight of the animal, the amount of cyanate per 100 g. of fluid should be 13 mg. In all these experiments the same pure preparation of potassium cyanate ("Kahlbaum") was used, but it cannot be denied that sodium cyanate would have been preferable if it had been possible to procure this in the same purity. However, it is out of the question that the animals may have been killed by the quantities of potassium ions applied simultaneously, as much larger doses of KCl and of KNO_3 may be given intravenously [Hald, 1904] as well as *per os* [Norn, 1925], without causing death.

It is possible that the method used by Montgomery for the detection of cyanate in the blood is not specific, or at any rate may possibly give rise to the formation of products of decomposition if the plasma be allowed to stand for 24 hours at 43° with toluene, which cannot be considered as a reliable antiseptic[1]. It is also possible that the blood contains cyanate in another form or combination, whose qualities differ from those of cyanate dissolved in water or added to the blood from outside. Without such an assumption the experiments described above indicate that cyanate is not a precursor of urea, as assumed by Werner.

[1] Similar, but aseptic, experiments have shown that cyanate cannot be present in the blood in concentrations exceeding 10 mg. per 100 cc. of plasma [Nicloux and Welter, 1922].

SUMMARY.

The experiments described exclude the presence of cyanate in the blood in concentrations exceeding 0·1 mg. per 100 cc. of plasma.

Cyanate, given intravenously or *per os*, is toxic; hence cyanate, as present in ordinary solutions, cannot be considered as a precursor of urea.

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II. A MODIFICATION OF THE CALCIUM PECTATE METHOD FOR THE ESTIMATION OF PECTIN.

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In the accurate estimation of the pectin content of a solution, such as apple juice, by the calcium pectate method described by Carré and Haynes [1922] the presence of a substance giving with calcium a compound insoluble in dilute acetic acid introduces a serious difficulty. No pectin solution containing oxalates, for instance, could be estimated by direct precipitation with calcium chloride. On the other hand, the simple older method of precipitation by alcohol is exceedingly untrustworthy. If the imperfection of this method were solely due to the dragging down of other substances by the alcohol precipitate, the latter could be redissolved in water and the pectin estimated as calcium pectate. Carré and Haynes have shown, however, that the amount of pectin obtained by precipitation with alcohol is dependent upon dilution, and that in very dilute solutions precipitation does not take place.

In view of the fact that the alcohol method of estimating pectin is still used by many investigators[1], a further series of experiments has been carried out in order to confirm the results obtained with this method by Carré and Haynes. These are here described, together with a modification of the Carré-Haynes calcium pectate method involving a preliminary precipitation of the pectin by acidified alcohol. This method may be used when estimation by direct precipitation with calcium chloride is impossible, as in the case of extracts made with ammonium oxalate.

[1] Wichmann [1922, 1923] recommends a method of pectin estimation by precipitation with alcohol, the precipitate being finally washed with 80 % alcohol. It has been proved repeatedly, however, that alcohol of this dilution effects ready solution of the pectin precipitate [Carré and Haynes, 1922; and p. 10 of the present paper]. Wichmann, on the other hand, considers pectin in the most dilute solutions to be insoluble in alcohol, and supports his conclusions by demonstrating that a pectin solution which cannot apparently be precipitated by alcohol alone will immediately produce a precipitate on addition of sodium chloride. It is obvious, however, that this procedure does not support the contention that pectin is insoluble in alcohol, but merely shows that a colloidal solution of pectin in alcohol is precipitated by an electrolyte. A detailed criticism of Wichmann's conclusions will be published shortly.

Precipitation by 95 % alcohol.

Precipitations were carried out with pectin solutions of varying dilution, and the first estimations were made in the following way: 25 cc. of a pectin solution, made from crude dried pectin, were diluted with a measured volume of water in each case and precipitated with 100 cc. of 95 % alcohol. The solution was allowed to stand overnight and then passed through hardened filter papers, from which the precipitate could be scraped with ease. This method of removing the precipitate was employed since it was impossible to dry the filter paper, previously treated with alcohol, to constant weight at 100°, and the method of filtering directly into a Gooch crucible was extremely slow. After washing with alcohol, the precipitate was transferred to a weighed glass dish and dried at 100°.

Table I shows the results of two experiments carried out in this way, and it will be seen that the alcohol precipitates, in those dilutions from which an alcohol precipitate was obtained, are heavier than those obtained by the calcium pectate method. This was attributed to the presence of impurities, and the method of weighing the alcohol precipitate directly was therefore abandoned. In the third experiment of Table I, and in all subsequent experiments, the actual weight of pectin contained in the alcohol precipitates was estimated by dissolving the precipitate on the filter paper in hot water, and reprecipitating as calcium pectate.

In the first experiment "Certo," a commercial pectin solution (ash 2·6 %), supplied by the Douglas Packing Co., was used, and for the second and third the material was pectin from lemon pulp (ash 4·65 %). In each experiment a solution of pectin was made up of the required strength and 25 cc. used for each precipitation.

Table I. *Amount (g.) from 25 cc. solution.*

	Undiluted		Diluted with 25 cc. water		Diluted with 50 cc. water		Diluted with 75 cc. water	
Exp.	Ca salt	Alcohol	Ca salt	Alcohol	Ca salt	Alcohol	Ca salt	Alcohol
1	0·090	0·146	0·093	0·133	—	—	0·094	0·112
	0·096	0·149	0·092	0·134	—	—	0·096	—
2	0·0443	0·0527	0·0445	0·0519	—	—	0·0443	No ppt.
	—	0·0515	—	0·0517	—	—	—	after standing a week
3	0·0456	0·0441	—	0·0400	—	0·0309	—	No ppt.
	0·0457	0·0440	—	0·0448	—	0·0290	—	after
	0·0458	—	—	—	—	—	—	standing 4–5 days

These experiments all serve to show the extreme untrustworthiness of alcohol precipitation, the two factors responsible for this being (1) dragging down of impurities by the alcohol precipitate, and (2) less complete precipitation with increasing dilution. At a concentration of about 0·04 % pectin is not precipitated at all by alcohol.

Precipitation with acidified alcohol.

In the course of experiments with apples on the conversion of the insoluble pectic constituent (which he calls protopectin) into soluble pectin, Fellenberg [1918] refers to the use of precipitation with acidified alcohol for the estimation of pectin in the presence of calcium salts. No details are given of the method of estimation, of the acid or concentration employed, or of the limits of accuracy of the method.

The following experiments were undertaken with a view of determining the effect of acidification upon the precipitation of pectin by alcohol. A preliminary experiment, conducted as Exp. 3 above, in which the pectin solutions were made up to $N/20$ with HCl before the addition of alcohol, indicated that acidification might lead to more complete precipitation. Acidification also greatly increased the rate of filtration of the alcohol precipitate, which in previous experiments was very slow. Experiments were next made to find out if at any concentration of acid there was a maximum precipitation, and whether this method of precipitation could be used as a preliminary to the calcium pectate method.

In all the experiments the alcohol itself was acidified, and, in order to avoid any effect due to the presence of a different amount of water in the alcohol at different concentrations of acid, the following procedure was adopted. For each estimation, 25 cc. of pectin solution were precipitated, the required concentration of acid being obtained by adding the necessary volume of 1·25 *N* HCl in 95 % alcohol, and making up the volume to 100 cc. with alcohol containing the same amount of water as the acid alcohol.

Table II. *Amount (g.) from 25 cc. solution.*

Conditions of precipitation		Exp. 1	Exp. 2	Exp. 3	Exp. 4
Directly as Ca salt		0·0322	0·0330	0·0330	0·0330
		0·0324	0·0330	0·0331	0·0330
N	HCl in alcohol	0·0329	0·0322	0·0335	—
		0·0326	0·0322	0·0335	—
N/10	,,	0·0323	0·0333	0·0335	0·0343
		0·0321	0·0330	0·0338	0·0337
N/25	,,	—	—	—	0·0335
		—	—	—	0·0318
N/50	,,	—	—	—	0·0332
		—	—	—	0·0334
N/75	,,	—	—	—	0·0328
		—	—	—	0·0325
N/100	,,	0·0325	0·0323	0·0326	—
		0·0321	0·0323	0·0334	—
N/1000	,,	0·0326	0·0328	0·0338	—
		0·0333	0·0321	0·0338	—
N/10,000	,,	0·0330	0·0317	0·0336	—
		0·0340	0·0316	0·0321	—
N/100,000	,,	Gelled	0·0329	0·0334	—
			0·0320	0·0339	—
Neutral alcohol		Gelled	0·0315	—	—
			0·0328	—	—

In Table II are given the results of representative series of experiments, made on electro-dialysed[1] "Certo" (ash 0·7 %). The acidity is that of the mixture, after the addition of alcohol. These series show surprisingly little difference in the amounts of pectin obtained by preliminary precipitation with alcohol in the presence of different concentrations of HCl, and there is reasonable agreement between the results so obtained and those obtained by direct precipitation as calcium pectate. The gel obtained in the first series in the case of precipitation without acid, and with $N/100{,}000$ HCl, was not repeated with fresh batches of material, although further experiments on the same material gave the same result. These results seem to point to the formation of a gel as an intermediate stage between solution and precipitation in the case of pectin, and acidification appears to aid flocculation [Haynes, 1914].

The results obtained by alcohol precipitation in the presence of the higher concentrations of HCl were shown by the above experiments to be in good agreement with those obtained by direct calcium pectate precipitation, and $N/10$ acid was the concentration selected for further experiments.

Washing the precipitate.

The results obtained by the use of acidified alcohol preliminary to precipitation as calcium pectate showed a slight tendency to be higher than those obtained by direct calcium pectate precipitation. This could not be due to the greater precipitation of impurities by the alcohol precipitate than by the calcium pectate precipitate, since the alcohol precipitate was redissolved and reprecipitated as calcium pectate. It was more probably due to the occlusion of the NaCl formed when NaOH was added to the dissolved pectin, which, having been previously precipitated by acidified alcohol, would contain HCl. The calcium pectate precipitate, although washed until the filtrate gave no precipitate with silver nitrate, and once reboiled, filtered and again washed, apparently still retained a small amount of NaCl. The following experiments on electro-dialysed material showed that this was actually the case.

In each experiment 25 cc. of solution were precipitated:

(1) directly as calcium pectate;

(2) directly as calcium pectate, a trace of pure NaCl being added to the pectin solution;

(3) by $N/10$ acid alcohol, hydrolysed with NaOH as in previous experiments—reboiled once;

(4) as in (3), but hydrolysed, after redissolving the alcohol precipitate, by KOH;

[1] One of us (A.M.E.) has worked out in detail a method for the purification of pectin based on the electro-dialysis method employed by Knaggs, Manning and Schryver [1923] for the purification of gelatin. A full account of this method will be published shortly.

(5) and (6) like (3) and (4) respectively, but reboiled several times after first washing free from chlorides. The results obtained were as follows:

No. of boilings	Ca pectate g.	Ca pectate and NaCl g.	Acid alcohol: hydrolysed by NaOH g.	Acid alcohol: hydrolysed by KOH g.
1	0·0313	0·0325	0·0322	0·0334
	0·0315	0·0322	0·0320	0·0320
3	—	—	0·0314	0·0313
	—	—	0·0313	0·0313

Careful washing of the calcium pectate precipitate is therefore necessary, and as continuous reboiling of the precipitate introduced the possibility of loss, the following experiments were made to determine (*a*) whether loss does occur when a calcium pectate precipitate is continuously boiled and (*b*) whether washing the alcohol precipitate free from HCl with 95 % alcohol before redissolving makes unnecessary the reboiling of the calcium pectate precipitate.

The results given below indicate that reboiling has no effect on the weight of the calcium pectate precipitate[1] after the second boiling has removed all traces of $CaCl_2$, and that washing the alcohol precipitate with alcohol tends to dissolve the pectin.

(1)

No. of boilings	1	2	3	4	5	6
Weight of Ca salt (g.) from 25 cc. solution	0·0349	0·0335	0·0336	0·0334	0·0334	0·0337
	0·0345	0·0336	0·0336	0·0332	0·0334	—

(2) *Weight of calcium pectate (g.) obtained by precipitating 25 cc. of solution.*

Directly as calcium salt	By alcohol not washed at alcohol stage	By *N*/10 acid alcohol washed at alcohol stage
0·0313	0·0291	0·0302
0·0314	0·0286	0·0295

Time of standing of alcohol precipitate.

In all of the foregoing experiments, the alcohol precipitate was allowed to stand overnight before filtration. This made the method slow but the following experiment, in which the precipitate stood for only an hour, indicated that prolonged standing was necessary for complete precipitation.

25 cc. of solution estimated.

Directly as calcium pectate g.	By *N*/10 acid alcohol after standing 1 hour g.
0·0263	0·0246
0·0262	0·0250

[1] Wichmann [1923] criticises the Carré-Haynes method of estimation on the grounds that there is great difficulty in removing traces of $CaCl_2$ from the calcium pectate precipitate, and that the excessive washing thus entailed may bring about re-solution. The results quoted above afford a satisfactory answer to these criticisms.

Method for estimation of pectin.

The method of estimation adopted as a result of these investigations is therefore as follows.

A volume of solution containing about 0·02 g. pectin is precipitated with four times its volume of alcohol, containing the amount of HCl required to make the resulting mixture $N/10$. After standing overnight, the precipitate is filtered, washed once with acidified alcohol and dissolved off the filter paper with hot water. It is then hydrolysed with NaOH and estimated as calcium pectate, the precipitate being washed free from chlorides and reboiled with water three times before being filtered into a Gooch crucible and dried at 100°.

The following experiments show the application of the method to pectin solutions of varying concentration:

Amount (g.) from 25 cc. solution.

I		II		III	
Calcium pectate	Acid alcohol	Calcium pectate	Acid alcohol	Calcium pectate	Acid alcohol
0·0466	0·0462	0·0250	0·0249	0·0134	0·0134
0·0465	0·0466	—	0·0249	0·0128	0·0136
—	0·0465	—	0·0249	—	0·0131
—	0·0462	—	—	—	0·0134
—	0·0456	—	—	—	0·0128
Mean 0·0466	0·0463	0·0250	0·0249	0·0131	0·0133

Amount (g.) from 100 cc. solution.

	IV	
	Calcium pectate	Acid alcohol
	0·0117	0·0108
	0·0114	0·0112
	—	0·0112
	—	0·0110
	—	0·0104
Mean	0·0115	0·0109

It will be seen that there is very good agreement between the two methods for pectin solutions as dilute as 0·05 %, but in the case of more dilute solutions the acid-alcohol method gives slightly lower results.

The application of the method to the estimation of pectin in the presence of oxalates, or other substances giving insoluble calcium salts, is quite satisfactory, as is shown by the following experiments. In each case 25 cc. of a 0·1 % pectin solution were used, 2 cc. of $N/10$ oxalic acid being added before precipitation of the pectin with acid alcohol.

Amount (g.) from 25 cc. solution.

	Exp. 1		Exp. 2	
	Calcium pectate (no oxalate)	Acid alcohol + oxalic acid	Calcium pectate (no oxalate)	Acid alcohol + oxalic acid
	0·0230	0·0239	0·0246	0·0248
	0·0235	0·0236	0·0246	0·0244
	—	—	—	0·0240
Mean	0·0233	0·0238	0·0246	0·0244

Pectin precipitation using acids other than hydrochloric.

In view of the activity of the hydrogen ion in causing the precipitation of oppositely charged colloids, it appeared very probable that the large increase in hydrogen ion concentration in the case of alcohol acidified with HCl was responsible for the quantitative precipitation of pectin by acidified alcohol. A similar increase in the precipitating power of alcohol with increased hydrogen ion concentration was found by Pauli [1922] in the case of albumin and glutin although in this case a maximum of precipitation occurred at the isoelectric point, and a further increase in hydrogen ion concentration caused a decrease in the amount of precipitation obtained.

The following experiments, in which a weak acid (acetic) and another strong acid (sulphuric) were substituted for hydrochloric acid, supported the view that, in the presence of a sufficiently large concentration of hydrogen ions (as with $N/10$ HCl and $N/10$ H_2SO_4), pectin is quantitatively precipitated by alcohol, whereas if this concentration is not high enough (as with N and $N/10$ acetic acid) precipitation is not quantitative.

Amount (g.) from 25 cc. solution.

No.	Calcium pectate method	Acetic acid N in alcohol	Acetic acid $N/10$ in alcohol	HCl $N/10$ in alcohol	H_2SO_4 $N/10$ in alcohol
I	0·0259	0·0240	—	—	—
	0·0264	0·0244	—	—	—
II	0·0259	—	0·0247	—	—
	0·0256	—	0·0247	—	—
III	—	—	—	0·0238	0·0240
	—	—	—	0·0240	0·0241

SUMMARY.

Further evidence is presented, showing that precipitation of pectin by neutral 95 % alcohol is an exceedingly untrustworthy method of estimation owing to the failure of alcohol to precipitate a dilute solution.

Acidified alcohol precipitates pectin completely at practically all dilutions. This precipitate does not give directly a measure of the pectin content of the solution, owing to the difficulty of washing the precipitate free from HCl without bringing about solution of the pectin. In the case of pectin solutions containing substances precipitable by calcium, such as oxalates, it has been found very convenient to precipitate first with acid alcohol, and then after washing the precipitate with more acidified alcohol, to dissolve it and reprecipitate as calcium pectate.

The authors wish to express their gratitude to Dr Haynes for her valuable criticism and advice.

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III. A NOTE ON THE HYDROLYSIS OF CYANIC ACID.

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In the liquid state below zero, or in aqueous solution, free cyanic acid may be regarded as an equilibrium mixture of the two components:

$$\underset{\text{(Enol-form)}}{HO.CN} \rightleftarrows \underset{\text{(Keto, or } iso\text{-form)}}{HN:CO.}$$

The composition of this mixture, according to Werner [1913, 1918], is determined by the temperature.

As is well known, free cyanic acid is unstable in aqueous solution, and readily undergoes hydrolysis with the production of ammonia and carbon dioxide; if the cyanic acid be in excess, a series of consecutive reactions follows, leading to the formation of urea, biuret, and polymers of cyanic acid [Werner and Fearon, 1920].

The initial stage in the hydrolysis, involving the hydration of the molecule, does not appear to have been investigated, for it is not known if both isomerides of the acid are equally susceptible to hydrolysis, or if only one is actually decomposed while the other undergoes a continual reversion in order to maintain equilibrium.

Of the two isomerides, it may be concluded that the keto-acid is the stronger. Isocyanic esters are known, but the enol or normal esters are only known in polymeric form. Also, union of ammonia with enol-cyanic acid produces ammonium cyanate, a compound easily dissociated; whereas, union of ammonia and keto-cyanic acid produces the much more stable urea.

A consideration of the structure of the two isomerides suggests that the enol-form, HO.CN, would resist the attachment of an additional hydroxyl group to the carbon atom to produce the isomeride of carbamic acid:

$$\begin{matrix} HO \\ HO \end{matrix}\!\!\diagdown\!\!\diagup C:NH.$$

On the other hand, the keto-form, HN : CO, might be directly hydroxylated to give $H_2N—CO—OH$ which is the formula usually ascribed to carbamic acid.

Solutions of cyanic acid undergoing hydrolysis at low temperature were tested for the presence of carbamic acid by a method suggested by Lewis and Burrows [1912].

Now, although carbamate can be unquestionably shown to be present during the hydrolysis of cyanic acid, its production is capable of several explanations. It may arise directly from cyanic acid in the manner indicated; or it may come from the ammonium carbonate produced during the hydrolysis, as shown by Nolf [1897]; or it may owe its origin entirely to the methods of analysis, unless the special precautions adopted by Macleod and Haskins [1905] are taken.

Faurholt [1921] quoted by Mack and Villars [1923] has found that under equilibrium conditions the proportion of carbonate to carbamate in an ammoniacal solution is about four to one. Consequently, if the carbonate and carbamate be determined simultaneously in the solution, the proportions found, according as they are below or above the ratio 4 : 1, will show if carbamate is being transformed into carbonate or if the reverse change is in progress.

The difficulty arising out of the possible confusion between carbamate and bicarbonate can be overcome by allowing the hydrolysis of the cyanic acid to proceed until the solution has become definitely alkaline owing to liberation of ammonia, and by using an excess of barium hydroxide for the precipitation of the carbonate; under these conditions it can be shown that a bicarbonate added to the solution is rapidly precipitated as barium carbonate.

Experimental.

Solutions of $N/5$ cyanic acid were prepared by treating 100 cc. of $N/2{\cdot}5$ potassium cyanate at 5° with an equal volume of $N/2{\cdot}5$ nitric acid. The cyanate solution was freshly prepared from the pure salt, using distilled water which had been boiled to expel carbon dioxide.

The potassium cyanate was prepared by the action of alcoholic potassium hydroxide on urethane (Kahlbaum). It contained only a trace of ammonium carbonate and, it may be noted in passing, was non-toxic when administered in doses of 0·1 g. to a rat and a rabbit. The solutions were kept at 5° before and after mixing, which was carried out rapidly.

At stated intervals, 25 cc. samples were drawn off, treated with a known excess of saturated barium hydroxide solution (50 cc.) and rapidly filtered. The residue, washed free from hydroxide, was dissolved in excess (50 cc.) of $N/10$ HCl and the carbonate determined by back titration with $N/10$ NaOH.

The filtrates, collected in small flasks free from CO_2, were incubated at 40° for 2 hours, by the end of which time any carbamate present had been hydrolysed to carbonate.

The barium carbonate thus formed was filtered off and estimated as before. The filtrates were again incubated to determine if all the carbamate had been hydrolysed. No further precipitate appeared in the solutions on standing for 2 hours, and a control solution of barium cyanate kept under the same conditions was also free from precipitate.

To determine if loss of carbon dioxide from the cyanic acid solutions at 5° constituted a serious error, one of the experiments was carried out in a Folin aeration apparatus, the air wash-bottle being filled with a 5 % solution of NaOH. After addition of the barium hydroxide solution the air in the apparatus was drawn over into a second wash-bottle containing a solution of barium hydroxide. It was found that no appreciable amount of carbon dioxide escaped from the cold solutions during the period of the experiment.

Hydrolysis of N/5 cyanic acid at 5°–7°.

Time mins.	Cyanic acid mg. %	Carbamic acid mg. %	Carbonic acid mg. %
0	860	0	0
10	688	—	—
40	452	—	211
60	—	82	214
120	—	82	226

The cyanic acid concentration of the solution ceases to bear a direct relationship to the rate of formation of carbonic acid as soon as the ammonia in the solution has reached a maximum and has begun to remove the free cyanic acid by fixing it in the form of urea.

The amount of carbonic acid formed in the early stages of the hydrolysis depends largely on how the cyanic acid is liberated. If the potassium cyanate solution be slowly added to the acid, the ammonium salt of the acid will be the chief product, since the cyanic acid is being completely hydrolysed as fast as it is liberated, in accordance with the equation:

(*a*) $HOCN + HNO_3 + H_2O = NH_2HNO_3 + CO_2$.

If, however, the acid be slowly added to the equivalent quantity of cyanate, urea will be the principal product owing to the interaction between the ammonia formed by hydrolysis and the excess of cyanic acid present:

(*b*) $2HOCN + H_2O \rightarrow HOCN + NH_3 + CO_2 \rightarrow NH : C\begin{matrix}NH_2\\OH\end{matrix} + CO_2$.

In the above result, the carbamic acid concentration is greater than would be required were it formed from ammonium carbonate as an equilibrium component. From this we conclude that carbamic acid represents the first stage in the hydrolysis of cyanic acid, and furthermore that it is probably formed by the direct hydration of cyanic acid in the keto-form, the interaction being exactly analogous to the interaction between cyanic acid and ethyl alcohol in the formation of urethane.

Carbamic acid is less stable than cyanic acid as shown by the behaviour of the salts and the ease with which the free acid is hydrolysed [Faurholt, 1921], hence its production as an intermediary is in conformity with Ostwald's [1902] law of successive reactions.

Accordingly, we represent the hydrolysis of cyanic acid as follows:

$$HO.CN \rightleftarrows HN : CO + H_2O \rightarrow \begin{matrix}NH_2\\OH\end{matrix}\!\!>\!CO \rightarrow \begin{matrix}OH\\OH\end{matrix}\!\!>\!CO + NH_3.$$

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IV. THE EFFECT OF CHEMICAL PRESERVATION OF EGGS UPON THE STABILITY OF THEIR VITAMIN CONTENTS.

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(*Received November 16th, 1925.*)

Chinese preserved eggs, or "pidan," offer an unusual opportunity for the study of some of the conditions under which the stability of vitamins may be affected. The preserved eggs are more or less chemically changed products of fresh ducks' eggs which are presumably rich in vitamin A, vitamin B, and the anti-rachitic food factor which exhibits certain vitamin-like properties. It should therefore be of interest to know what effects the preserving agents and the chemical changes have upon the stability of these vitamins. Furthermore, such a study would at the same time disclose the vitamin value of "pidan" as a food. This is of practical significance in China where the preserved egg is perhaps as much used on the table as is cheese in Western countries.

The method of preservation according to the Chinese Economic Bulletin is the following.

Fresh ducks' eggs are selected and washed. For the preservation of 100 eggs, mix 5 ounces of pure soda, 25 ounces of straw ash, and a little less than 4 ounces of table salt with about 20 ounces of boiling water to give a uniform mixture. Then add gradually 40 ounces of slaked lime to make a thick paste. Apply heat if necessary and keep stirring until the ingredients are thoroughly mixed. A layer of this mixture about a quarter of an inch thick is wrapped round each egg and covered with rice husks to prevent sticking. The eggs are then carefully laid in earthenware jars which are sealed with wet clay when full. In about a month the eggs acquire the desired flavour and degree of coagulation and are ready for the table.

"Pidan" looks quite different from the fresh egg. The white has not only coagulated but turned dark brown and translucent, not unlike coffee jelly in appearance. The coagulated yolk looks deep green or greenish grey with concentric rings of different shades of grey, brown or dark green. The egg has a peculiar piquant lime taste and a decidedly ammoniacal odour, especially when freshly opened.

Blunt and Wang [1917] some time ago analysed these eggs and found that there is a marked increase in the ash content and in the alkalinity of the ash, and a partial decomposition of the proteins and phospholipins resulting in an excessive production of free ammonia and in a diminution of the yolk-fat. According to these authors, the characteristic changes in the eggs are brought about by the combined action of bacteria and enzymes, as well as by the alkaline preservative.

Experimental.

I. *Vitamin A.*

Exp. 1. In a preliminary test the results of which are presented in Fig. 1, it was found that the addition of 20 % of the preserved egg-yolk to a basal ration deficient in vitamin A, on which four young rats had declined in weight and developed xerophthalmia, induced promptly the return of vigorous growth and the cure of the eye condition. The two controls continued to decline.

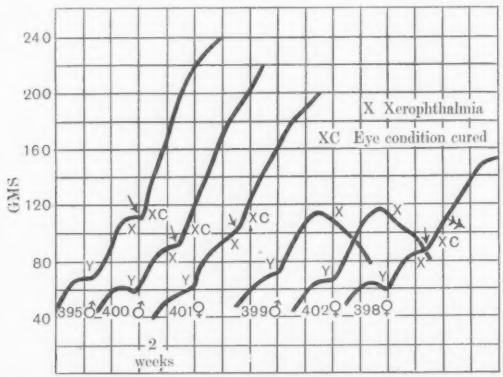

Fig. 1. Ration consisted of caseinogen 18, corn-starch 72, yeast 5, and salt mixture 5. At the position of *Y* the amount of yeast was doubled. Arrowhead indicates the addition of 20 % of the yolk of the Chinese preserved egg, and double arrowhead the withdrawal of the yolk.

Exp. 2. Eight albino rats 4 weeks old weighing on the average 43 g. were placed on a basal ration of caseinogen 20, corn-starch 65, yeast 10, and McCollum's 185 salt mixture 5. The caseinogen, starch, and yeast had been purified by oxidation and tested to be free from vitamin A but satisfactory in other respects.

After the initial growth, which lasted from 6 to 8 weeks, all animals except one ceased to gain and towards the end of the 8th week three developed xerophthalmia. At this point half of the animals, including one with sore eyes, were put on the basal ration plus 5 % of the preserved egg-yolk, incorporated into the basal ration paste. The other four rats were placed in a similar manner on 5 % of liquid yolk from fresh ducks' eggs. The feeding was continued for

4 weeks. The results are shown in Figs. 2 and 3 respectively, from which it will be seen that there was an immediate response to the addition of the preserved yolk as well as the fresh yolk as indicated by the prompt return of vigorous growth and the cure of the eye condition in both groups of animals.

Exp. 3. Four young rats, which had declined in weight on the basal ration deficient in vitamin A, were fed 3 % of the preserved egg-yolk. The response was quite as definite though not so immediately apparent as by adding 5 % of the preserved yolk.

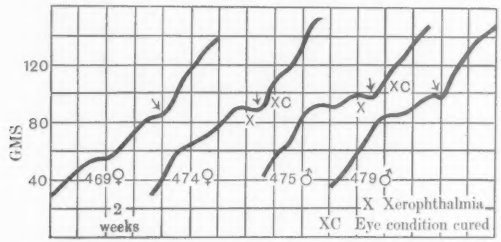

Fig. 2. Diet consisted of caseinogen 20, corn-starch 65, yeast 10, and salt mixture 5. At the point of arrowhead 5 % of preserved egg-yolk was added.

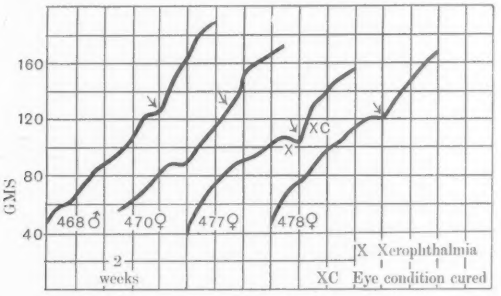

Fig. 3. Ration as in Fig. 2. Arrowhead indicates the addition of 5 % of liquid yolk from fresh ducks' eggs.

Exp. 4. Six rats 4 weeks old were placed on the same basal ration as used in previous experiments. In the 8th week, when growth in most of the animals had ceased, three were given, in addition to the basal ration, 2 % of oxidised ether extract of dried preserved egg-yolk[1]. In the 10th week the other three rats received, in addition to the basal ration, 2 % of the same lot of ether extract, but not oxidised. The experiment was terminated at the end of 12 weeks of feeding. The results are presented in Fig. 4, from which it will be seen that those rats receiving the oxidised ether extract from the beginning of the 9th week continued to lose weight and developed xerophthalmia, and one

[1] The preserved egg-yolk had been dried in an oven at 45° for three days and next extracted with ether in a Soxhlet, the ether extract had then been exposed to an air-bath at about 120° for six hours.

died before the 12th week. On the other hand, the other three animals immediately responded to the addition of 2 % of non-oxidised ether extract as shown by the return of good growth and the improvement in general well-being of the animals.

From the results of the foregoing experiments we may conclude that the chemical changes occurring in the Chinese preserved eggs have little or no deleterious effect upon the stability of vitamin A.

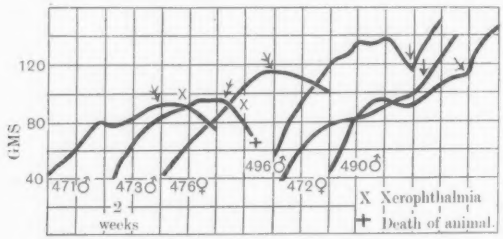

Fig. 4. Diet consisted of caseinogen 20, starch 65, yeast 10, and salt mixture 5. At the position of single arrowhead 2 % of the ether extract of preserved egg-yolk was added. Double arrowhead indicates the addition of 2 % of oxidised ether extract.

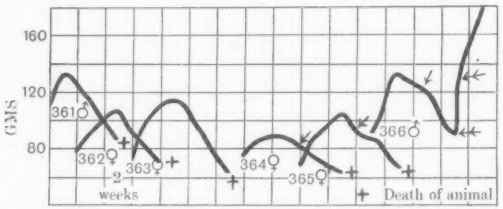

Fig. 5. Diet consisted of caseinogen 18, corn-starch 67, fresh butter-fat 10, and salt mixture 5. At the point of arrowhead 25 % of the preserved egg-yolk was added. During the 12-hour period marked between the double arrowheads, 10 g. of liquid yolk from fresh ducks' eggs was fed.

II. *Vitamin B.*

Exp. 1. Six young rats which had grown vigorously on a normal diet were transferred to the following basal ration deficient in vitamin B: caseinogen 18, corn-starch 67, fresh butter-fat 10, and McCollum's 185 salt mixture 5. Beginning in the 4th week when all the rats had declined in weight, three were fed, in addition to the basal ration, 25 % of the preserved egg-yolk. As illustrated in Fig. 5, these three rats continued to lose ground and two died about 3 weeks later. The third rat would also have succumbed but for a change made in its diet. This consisted of a single feeding of about 10 g. of fresh ducks' egg-yolk. Over-night the animal gained 34 g. and began to eat more than usual amounts of the preserved yolk and basal ration mixture. During the following 9 days another increment of 50 g. in weight was added. The three control rats on the basal diet alone lost weight rapidly after the initial growth of 1 to 3 weeks' duration. One died in the 5th week, another in the 6th, and the third in the 7th week.

This experiment indicates that the chemical changes in the preserved eggs are quite unfavourable to the stability of the growth-promoting vitamin B. Apparently it is the presence of alkaline products in the preserved eggs that is destructive of the originally rich content of vitamin B in the fresh ducks' eggs, since it is a well-known fact that vitamin B is very sensitive to alkaline reagents.

III. *The anti-rachitic food factor.*

Exp. 1. Sixteen albino rats 4 weeks old were placed on Sherman and Pappenheimer's 84 basal rachitic diet, which consists of patent flour 95, calcium lactate 2·9, sodium chloride 2, and ferric citrate 0·1. At the end of 3 weeks when the animals should all have developed rachitic lesions in the bones they were divided into 4 groups of 4 each. To the basal ration of one group was added 5 % of the preserved egg-yolk, of another group 5 % of oxidised preserved yolk, of a third group 5 % of fresh ducks' egg-yolk, and of a fourth group 5 % of olive oil. Two weeks later the animals were killed under ether and X-rayed. The X-ray examination showed that healing of rachitic bone lesions had occurred in the rats which received either the fresh yolk, the preserved yolk, or the oxidised yolk. Healing was, however, most pronounced in the rats receiving fresh yolk and least marked in the animals fed the oxidised preserved yolk. The control rats taking olive oil all showed rachitic lesions of a severe grade without presenting any evidence of healing.

Exp. 2. In this experiment the anti-rachitic property of the ether extract and the oxidised ether extract of preserved yolk was tested. This ether extract had been found to contain vitamin A but the oxidised product to be free from vitamin A. The results and other details are presented in Table I.

Table I. *The anti-rachitic potency of the ether extract from the yolk of Chinese preserved eggs.*

Ration	Pre-experiment diet: Milk Millet Butter	Pre-experiment diet: Soybean Millet Corn Cabbage	Age of rats Days	Duration of feeding Weeks	Initial wt. g.	Final wt. g.	Results: X-ray examination	Results: Microscopic examination
1. Basal plus 2 % ether extract	R. 378	—	24	3	40	52	No rickets	No rickets
	R. 379	—	24	3	30	37	,,	,,
	—	R. 382	30	3	40	62	,,	,,
2. Basal plus 2 % oxidised ether extract	R. 376	—	24	3	34	42	? Slight rickets	No rickets
	R. 377	—	24	3	34	43	,,	,,
	—	R. 381	30	3	42	60	Rickets	Slight rickets
3. Basal	R. 380	—	24	3	34	42	Rickets	Rickets
	—	R. 383	30	3	33	44	Severe rickets	Severe rickets
	—	R. 384	30	3	44	58	,,	,,

These experiments show that the anti-rachitic food factor like the growth-promoting and anti-xerophthalmic vitamin or vitamin A is not affected to any appreciable extent by the chemical changes in the preserved eggs. That the oxidised ether extract was found to be less effective in preventing rachitic

bone lesions might be due to factors other than oxidation, since it has been shown by Hess and Weinstock [1924] that even drying the yolk (of hens' eggs) and keeping it in a dried state would markedly reduce its anti-rachitic value.

Summary and Conclusion.

"Pidan" is made from raw ducks' eggs by applying a mixture of slaked lime, straw ash, soda, table salt, and water.

A study of the stability of the vitamins in these preserved eggs has shown that the originally rich vitamin B content is practically completely destroyed but that the potency of vitamin A and the anti-rachitic food factor is little or not at all affected.

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V. THE NATURE OF THE ACTION ON A PHOTOGRAPHIC PLATE OF SAWDUST AND CHOLESTEROL IRRADIATED BY A MERCURY VAPOUR QUARTZ LAMP.

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(*Received November 18th, 1925.*)

This work was undertaken as the result of observations by Hume and Smith [1924] indicating that pine sawdust which had been irradiated by a mercury vapour quartz lamp developed anti-rachitic properties. For some time it was supposed by them that the activity of the irradiated sawdust might be due to secondary radiations or some emanations given out by the particles of wood exposed to ultra-violet rays.

This view was countenanced by the statement of Kugelmass and McQuarrie [1924] that anti-rachitic substances, *e.g.* cod-liver oil, egg-yolk and sperm oil, were capable of fogging a photographic plate even when the plate was exposed through a quartz cover sealed in such a way as to preclude the action of a volatile substance.

That certain animal and vegetable substances are capable of producing fog on a photographic plate is not new. Russell [1906] studied the phenomenon and showed that the action was intensified by a previous exposure of the substance to sunlight or a carbon arc. Temperature increased the effect.

The substances with which Russell worked were woods of various kinds, coal, wool, fur and feathers. He observed an effect whether the plate was in contact with the substance or at a distance from it, or when air was drawn over the substance on to the plate. He also found that, given sufficient exposure of the plate, whatever produced the fogging could travel round the edges of an opaque mask, and that glass and mica prevented its passage. He believed the effect to be due to the evolution of hydrogen peroxide.

Experiments with pine sawdust.

These confirm the conclusions of Russell.

Sawdust in a glass dish without a cover was exposed to a 4 amp. mercury vapour quartz lamp at 15 inches for 15 minutes. The glass dish was taken to the dark-room and a photographic plate (Eclipse orthochromatic superspeed, as used throughout these experiments) was laid, sensitive side downwards on the sawdust. Between it and the dust was placed a piece of thin zinc sheet covered with black plate-wrapping paper. The whole was then placed in a light-tight box, wrapped in a photographer's black cloth, and kept in a room

at 37° for six hours. After development with pyro-metol developer for 5 minutes at room temperature, an image of the zinc plate was found, and a pattern of the grains of sawdust where the zinc was not opposed. In the negative the granules showed black and the spaces between white.

The experiment was repeated with the plate inclined at an angle. The exposures and development were the same as in the previous experiment. A pattern of the sawdust was again visible where contact of the sawdust and plate existed, but on the parts of the plate apart from the sawdust, fogging, diminishing in density as the distance between the plate and sawdust increased, was alone visible.

A Petri dish of sawdust without a lid was irradiated under a mercury vapour quartz lamp for 15 minutes and placed on a block of paraffin wax. A quartz Petri dish lid was then warmed and pressed down over the sawdust, so that it melted the paraffin wax, and its edge was firmly embedded all round. Any places which did not seem to be air-tight, were made so by means of a hot knife. The photographic plate was fastened down on to the quartz plate with an elastic band, a strip of black paper being placed between in order to show an image. The whole was placed in a light-tight box and then in the warm room (37°) for 6 hours. On development there was no sign of fogging. The experiment was repeated with an exposure of 18 hours of the plate to the substance, with the same result.

Air was sucked slowly over a covered dish of sawdust and through a blackened tube so as to impinge upon a plate protected from the light by a blackened bell-jar. The suction was continued for 24 hours, and the plate then developed. It showed a patch of fog under the inlet tube the border of which was a sharp line on the side nearest the inlet, the patch extending in a fan-shaped outline of diminishing intensity towards the outlet tube, showing that the current of air sucked the emanation from the sawdust in the direction of the draught.

The same experiment was repeated having a Richardson's gas-washing bottle containing potassium iodide starch solution to receive the air from the sawdust instead of the photographic plate and bell-jar. In 20 minutes there was a tinge of blue visible, and ultimately the liquid became the typical starch iodine blue.

Experiments were also carried out with fur, skin, feathers and wool. In these the irradiated substances were fastened to the plate with elastic bands.

With the exception of a rather doubtful result from white wool knitted into a pattern, no effect was produced on the plates.

These observations show that, provided the possibility of a vapour passing from the sawdust to the plate is excluded, no photographic effect occurs, or in other words, secondary radiations capable of traversing quartz are not produced.

Russell's view that the action is due to the formation of hydrogen peroxide or ozone is supported.

Experiments with cholesterol.

In view of the observations of Steenbock [1925] and Hess and Weinstock [1925] on the use of irradiated cholesterol as an anti-rachitic, similar experiments were made with cholesterol.

Cholesterol was gently melted on a roughened glass plate, and after irradiation placed in contact with a photographic plate. The fog produced was intense. When however the plate was separated by quartz, as in the third experiment above, no fogging occurred.

Conclusions.

1. The action on a photographic plate of sawdust after irradiation by ultra-violet rays is not due to any secondary radiations given out by it, which are capable of passing through a plate of quartz.

2. The action appears to be due to the production and subsequent gradual emanation of either hydrogen peroxide or ozone.

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VI. THE USE OF COLLOIDAL FERRIC HYDROXIDE SOL FOR ADSORBING VITAMINES B AND D[1].

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(*Received November 23rd, 1925.*)

Eddy, Kerr and Williams [1924] have described the preparation and isolation of vitamine D (yeast-growth-promoting vitamine) using the following method. An aqueous extract of yeast, freed from proteins by coagulation, was concentrated to suitable strength, the p_H of the solution adjusted to 4·7 and one volume of a colloidal ferric hydroxide sol added. The ferric hydroxide was prepared according to the method described by Nelson and Kerr [1924]. It was claimed that under such conditions impurities are eliminated without impairing the activity of vitamine D. After freeing the liquid from this first precipitate and adjusting the reaction to p_H 5·3, 4 volumes of the same iron sol were added; it was stated that almost all the vitamine D could be obtained in this way. In looking over the curves of adsorption of vitamine D at various hydrogen ion concentrations one is struck, however, by the divergence of the results of the third parallel experiment.

Before going into the actual isolation of vitamine D we have made a thorough study of the method described. As a starting point we have used aqueous and alcoholic extracts of yeast, wheat bran and rice polishings. We do not wish to cast the slightest doubt on the results of Eddy, Kerr and Williams, but only to point out that their method did not yield good results in our hands. In our study we have extended our procedure to vitamine B and we have studied the adsorption of both substances at various hydrogen ion concentrations. Our results suggest perhaps that the method of precipitation of vitamines B and D by means of colloidal iron sol yields results which are not constant and which cannot be relied on.

As a starting material we prepared aqueous extracts of yeast, and aqueous and alcoholic extracts of wheat bran and rice polishings, in which the vitamine D content has been determined by the method of Funk and Dubin [1920]. This method has been uniformly used throughout the whole investigation. For the determination of vitamine B we used pigeons which had been placed in vitamine B equilibrium by the method of Funk and Paton

[1] In this paper the author uses his own system of nomenclature, which is not that usually adopted in this country. (Ed.)

[1922] and Collazo and Funk [1924, 1925]. In this way the amount in cc. of each preparation given per day was determined which sufficed to keep the animals at constant weight on a diet of polished rice. By substituting the original solution by the filtrates obtained after precipitation with colloidal ferric hydroxide sol, the loss or gain in weight of pigeons per 100 g. of weight and per unit of vitamine given was taken as an index of the vitamine content. This method has been found to give quite reliable results. In our investigation we did not limit ourselves to the two hydrogen ion concentrations but we have used a whole scale from p_H 4·0 to 5·5 which has been adjusted both by the colorimetric method of Sörensen and by the gas chain method. Aside from these systematic adsorption studies we have repeated the work of Eddy, Kerr and Williams on the isolation of vitamine D by keeping exactly to their method. Here also we were unable to obtain a noticeable precipitation of vitamine D. After these negative results we tested whether this method could be adapted for the preparation of vitamine B. By using larger volumes of colloidal ferric hydroxide sol we did not succeed in throwing down more than 30 % of the vitamine B present originally in the solution. Somewhat similarly irregular results were obtained by Levene and van der Hoeven [1925] using silica gel. The amount of vitamine B adsorbed varied from 41 to 65 % and the authors remark that the degree of adsorption depends on the presence of casual impurities, in their case probably of phosphates. Our results can be seen from the appended figures.

Discussion of the Results.

Vitamine D.

1. *Yeast.* In case of purified aqueous extract of yeast some of the vitamine D was adsorbed in the vicinity of p_H 5·3, whilst on a second precipitation the smallest amount of vitamine D was found in the filtrate of p_H 4·4 (see Fig. 1).

2. *Wheat.* In case of wheat the first precipitation gave a slight diminution of vitamine D at p_H 5·0, whilst the second precipitation gave the best adsorption in the vicinity of p_H 4·6 (Fig. 2).

3. *Purified alcoholic extracts of rice polishings.* Two experiments made with the same extract (shown in Figs. 3 and 4) gave a great deviation of results. The first precipitation gave a considerable loss of vitamine D, whilst the second gave an increased vitamine activity as measured by growth of yeast. In the second experiment (Fig. 4) the results were more regular and the vitamine D was mostly precipitated at p_H 4·6.

4. *Aqueous extract of rice polishings.* Here only the result of the second precipitation was recorded and, as we see from Fig. 5, practically no effect was noticed.

If we summarise the results of the experiments here recorded and others that we have made during our investigation we are unable to find that the

colloidal ferric hydroxide sol has any specific adsorbing power for vitamine D. Moreover our curves tend to show that the reading of the results is very much influenced by precipitation of inhibitory substances for yeast growth which are more or less adsorbed at various p_H values. One may find perhaps that the growth of yeast increased not because some of the vitamine D was adsorbed, but by elimination of some growth-inhibiting substances, whilst in other cases we might find an apparent diminution of vitamine D by the mere fact that the inhibitory substances happened to remain in solution. The method in any case was a failure in our hands, as the iron precipitates decomposed by the method by Eddy, Kerr and Williams failed to yield any vitamine D.

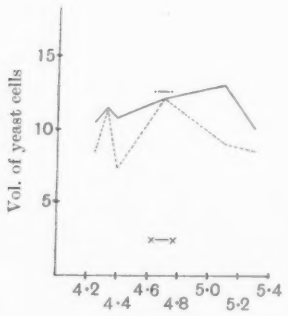

Fig. 1.

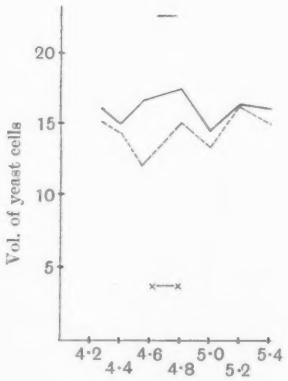

Fig. 2.

Fig. 1. Influence of hydrogen ion concentration on the adsorption of vitamine D by colloidal ferric hydroxide sol. Aqueous yeast extract used.

The upper short line indicates the activity of the extract before treatment, whilst the lower short line is the control with yeast cells without adding the extract. The continuous curve is the result of the first treatment with a single volume of the colloidal solution whilst the interrupted curve is the result of the second precipitation with four volumes of the same adsorbent.

Fig. 2. Same as fig. 1 using aqueous wheat bran extract.

Vitamine B.

Being unsuccessful with the preparation of vitamine D by the adsorption method, we turned our attention to vitamine B, which according to our previous findings is much more easily adsorbed than vitamine D. To study this question we have used the same extracts as above but we have investigated only the effect of the first precipitation. Working with alcoholic extracts of rice polishings the method was without value, the filtrate containing as much vitamine B as the original solution (Fig. 6). Somewhat better results were obtained with aqueous yeast extract. Here some adsorption of the substance could be recorded at p_H 5·1 but in the best case adsorption only to the extent of 30 % could be obtained. With an aqueous extract of rice polishings two minimal points could be recorded, one at p_H 4·2 and the second at p_H 5·0.

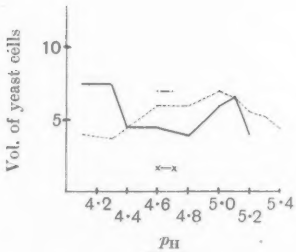

Fig. 3.

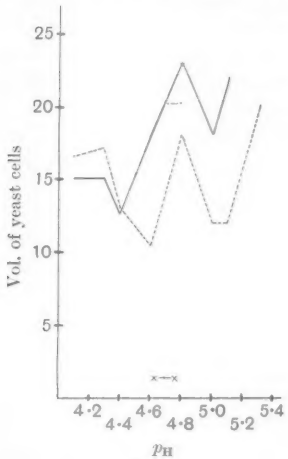

Fig. 4.

Fig. 3. Same as fig. 1 using alcoholic extract of rice polishings.
Fig. 4. Repetition of experiment recorded in fig. 3.

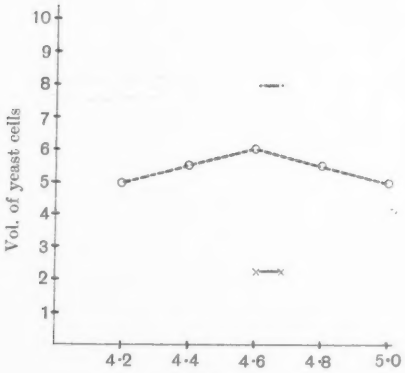

Fig. 5.

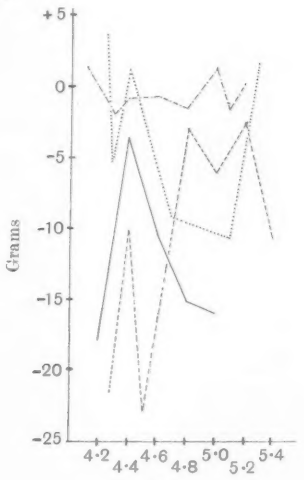

Fig. 6.

Fig. 5. Effect on vitamine D activity of one single addition of four volumes of colloidal ferric hydroxide sol to an aqueous extract of rice polishings.

Fig. 6. Effect of addition at various hydrogen ion concentrations of one volume of colloidal ferric hydroxide sol on the activity of vitamine B measured by the action of filtrates on pigeons.

The curves indicate the loss or gain in the weight of pigeons calculated on 100 g. of body weight and 100 cc. of each particular extract. The calculation of the amount of extract given is of importance because each bird received a dose which corresponded to its individual vitamine requirement.

——— aqueous extract of rice polishings.
– – – – – „ „ wheat bran.
—•—•—• alcoholic extract of rice polishings.
..... aqueous extract of yeast.

A similar effect was found by using an aqueous extract of wheat. Increasing the amount of colloidal ferric hydroxide sol to 4 volumes did not yield any more favourable results and the preparation of vitamine B from the precipitates obtained was on the whole unsuccessful.

SUMMARY.

The adsorption of vitamine D using the method of Eddy, Kerr and Williams (colloidal ferric hydroxide sol) gave unreliable results in our series of experiments. This method applied to preparation of vitamine B gave also negative and inconsistent results. It is probable that the degree of adsorption depends on the presence of casual impurities in the extracts.

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VII. THE VITAMIN B OF LEMON RIND.

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(*Received November 26th, 1925.*)

In a preliminary investigation it was shown that the outer rind of the fresh lemon (the flavedo) must contain considerable amounts of vitamins [Willimott and Wokes, 1925, 1, 2]. In the work described in these papers no attempt was made to characterise the vitamins present in the rind but here results are described which show that vitamin B is one at least of the vitamins concerned.

Most of the earlier work on the Citrus family has been devoted to the investigation of the juice rather than the peel; but as the more immediate questions were solved, attention was turned to the important problems presented by the peel. In this connection, the peel of the orange appears to have been investigated almost to the exclusion of that of the other members of the family, and so far as it is known, there is no record in the literature of work carried out on the rind of the lemon. From a study of the alcohol-ether extract of orange peel, Cooper [1920] concluded that this material must contain considerable amounts of vitamin A. Cooper also makes the interesting statement that similar experiments indicate the presence of vitamin A in the peel of the lemon and the grape fruit. Details of these observations were promised but further published records of this work have not been found. A résumé of work done on the Citrus family, with complete experimental details of the methods adopted, is given in the papers first cited, so that little need be said here. The present experiments were planned so that a group of albino rats received a diet adequate in every factor except vitamin B, which was supplied as an alcoholic extract of lemon rind. The growth curves thus obtained were compared with those of a control group receiving a complete synthetic diet.

Methods.

Inbred albino rats (*Mus norvegicus albinus*) of Wistar Institute origin were used in this investigation. These animals were in good condition at the time they were set up in experiment. The basal diet fed to both control and experimental groups was made up as follows:

Starch (pure potato)	40 %
Sugar (fine castor)	17
Oil (refined palm-kernel)	15
Protein (pure extracted caseinogen)	23
Salt mixture	5

Pure food products only were used and the usual precautions were observed, particularly with regard to the caseinogen, so as to render the basal diet vitamin-free.

For the salt mixture, Hopkins' mixture with some modifications was employed. Mn and F have been increased and Si and Al have been included as additional ions, so that the salt mixture had the following composition:

NaCl	51·8
$MgSO_4 . 7H_2O$	79·9
$NaH_2PO_4 . 2H_2O$	104·1
K_2HPO_4	286
$CaH_4(PO_4)_2$	162
Calcium lactate	390
Ferric citrate	35·4
NaF	0·25
$MnSO_4$	5·00
KI	1·00
Potash alum	0·025
Na_4SiO_4	1·00
	1116·5

The balanced ration so prepared had a nutritive ratio of 1 : 4 and an energy value of 467·5 calories per 100 g. It was thus adequate for the energy and protein requirements of the rat. For the controls the three vitamins were supplied daily as follows:

Vitamin A. Four drops of pure Norwegian cod-liver oil per head per diem.

Vitamin B. An extract of yeast, at the rate of 0·25 cc. per head per diem.

Vitamin C. The filtered juice of fresh lemons at the rate of 0·20 cc. per head per diem.

Distilled water in separate vessels was supplied to all the animals *ad lib.*

The experimental group was restricted to the basal diet with the addition of vitamin A, supplied by cod-liver oil at the same rate as for the controls. The only source of supply for vitamin B (possibly C also) was the extract of lemon rind, which was fed at the rate of 0·5 cc. per head per diem. At this level, this quantity of extract was equivalent to 0·25 g. of the rind. The lemon extract was made from the outer rind of the fresh lemon and in obtaining this material the directions of the British Pharmacopoeia [1914] for Cortex Limonis were followed. Cortex Limonis, B.P., is described as the fresh outer part of the pericarp of the fruit; the outer surface pale yellow and more or less rough, the inner surface should have very little of the white spongy portion of the mesocarp adhering to it. A transverse section shows a peel about 3 mm. thick, of which the outer 1 mm. is yellow and is termed the flavedo and contains numerous oil glands: the inner 2 mm. is white and is called the albedo.

Fresh lemons were first washed, dried and the rind carefully peeled off with a sharp knife so that only the flavedo was obtained. The rind was now cut into small pieces, placed in a wide-mouthed bottle and 90 % alcohol added at the rate of 2 cc. of solvent to 1 g. of rind. The bottle was well corked and shaken at frequent intervals for at least three days, sometimes longer, the extraction being carried out at room temperature. A cloudy liquid resulted which on filtering gave a clear, yellow solution possessing a pleasant aromatic odour. This was the extract fed to the experimental group as a source of vitamin B. Apart from any vitamin present, the oil thus extracted contains the terpenes *d*-limonene, α-terpinene and α-phellandrene, the first being present in largest amount and the three representing 90 % of the bulk of the oil; the aldehydes, citral (minimum 4 % of the oil) and citronellal; the ester geranyl acetate, and from Palermo lemons—employed in this work—linalyl acetate also. The alcoholic extract also contained some colouring matter, whilst a little mucilage was retained by the filter paper. Another important constituent of the peel, the glucoside hesperidin, is insoluble in alcohol and is therefore not present in the extract.

If vitamin B is present in the rind, a considerable amount will be extracted by 90 % alcohol. For the extraction of vitamin B, lower strengths of alcohol are more efficient and from the work of Funk, Harrow and Paton [1922], 70 % alcohol appears to be the optimum strength. Alcohol of 90 % strength was employed as solvent in order that any findings upon the extract could be applied to B.P. preparations in which lemon rind is used. If vitamin B is proved to be present in the extract the case for its presence in the rind itself is stronger, as the solvent employed would not extract all of this factor. The fact of the possible presence of vitamin C in the extract does not interfere with the test for vitamin B, as it is well known that vitamin C is not essential in the normal diet of the rat [Parsons, 1920]. Differences, therefore, in the growth rates between the control and experimental groups turned upon the vitamin B content of the extract.

The animals were fed side by side, under the same conditions. Sufficient of the ration was always provided so that a small amount remained over each day. The control group consisted of 5 male and 4 female albinos, the average weight of which at the beginning of the experiment was 79·5 g. The experimental group was made up of 3 males and 5 females of average weight 69·2 g. In the feeding of this group, the lemon extract was well mixed in with the basal ration before being given to the animals. The alcohol, at the level the extract was fed, was not observed to have any undesirable effect upon the animals. In the third week of the experiment, the daily consumption of food was ascertained by means of back weighings. Although the food was in the form of a dry powder, it was possible to obtain a reliable estimate by taking precautions against the loss due to scattering. Lipped pots, which helped to reduce scattering, were placed on wide metal trays which caught the small quantity of food actually spilled. This amount was carefully col-

lected and added to that remaining over in the feeding pots in the 24 hours for the back weighing. It was found that the food consumption of the controls was on the average equivalent to 48 cals. per 100 g. body weight, whilst that of the experimental set was approximately 46·7 cals. per 100 g. body weight; quantities ample for the calorific needs of the rat [*vide* Donaldson, 1924]. Moreover, the food equivalent of these energy consumptions supplied adequate amounts of protein. Both sets were weighed at the same time at intervals of three days.

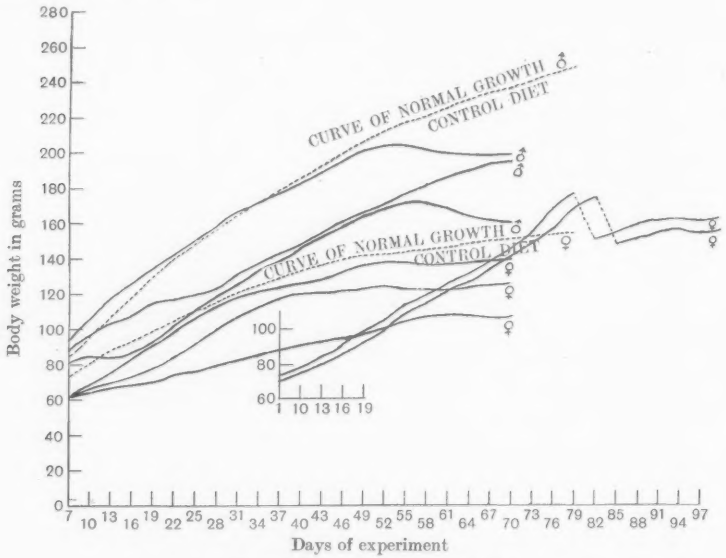

Fig. 1. Vitamin B of Lemon rind.

Growth Effects.

Each animal in the experimental group grew at a more or less satisfactory rate during the ten weeks of the experiment. There were no deaths. At the end of the experiment, the average of the males was 79 % that of the controls; in the females the average weight was 91 % that of the controls. The mean rate of growth for the whole group when the observations were discontinued was, therefore, not very much lower than that of the control animals. Moreover, the experimental group remained in good health throughout the period, and at the end, exhibited all the characters of normally nourished rats. There is little doubt that had the experiment continued, the animals would have reached the same weight as the controls although by a slower path. The possibility that the animals were obtaining their vitamin B requirement from the faeces, as shown by the work of Steenbock, Sell and Jones [1923], was excluded by the fact that faecal matter fell into trays and was so removed

from the cages. As the diet had proved satisfactory for growth, it was tested for its adequacy in reproduction and rearing. Two of the experimental does were mated with bucks from the same group. Two litters were produced containing 6 and 8 individuals respectively, but they were not reared in either case. The diet, therefore, whilst adequate for growth could not sustain successful rearing of offspring.

At the conclusion of the experiment, a buck and a doe were each chloroformed and autopsied. The internal organs were found to be normal in both cases. In the buck it is noteworthy that spermatozoa were isolated and recognised under the microscope.

Conclusions.

From the experimental results obtained, it is evident that vitamin B is present in appreciable amounts in the rind of the fresh lemon. When only 0·5 cc. (equivalent to 0·25 g. of rind) of the lemon extract was fed per head per diem as sole source of this vitamin, nearly normal growth resulted. As the albinos were making satisfactory progress, the supply of the extract was not altered, although with a slight increase, it seems probable that complete normal growth would have been obtained. The supply of vitamin B though sufficient for reproduction was inadequate for successful rearing. It is well known that, in contrast to vitamin A, at all periods absence or deficiency of vitamin B is speedily manifested [Osborne and Mendel, 1920, 1]. As growth was maintained for a period as long as ten weeks, it appears certain, therefore, that appreciable quantities of this vitamin were present in the extract. Rather more could be expected to be present in the rind as 90 % alcohol is not the optimum solvent for vitamin B. These results are in accordance with the findings of Osborne and Mendel [1920, 2], who demonstrated the presence of vitamin B in the albedo of orange peel. Particularly so, as it appears very probable that some contamination with the rind (flavedo) of the orange would be almost inevitable, since the preparation of the inner peel involves the removal of flavedo with unavoidable rupture of the oil glands. The likelihood of this interpretation appears to be supported by the quantities of albedo and flavedo respectively which are required by the animal for growth. Allowing for the fact that Osborne and Mendel worked upon the orange, it is striking that these authors used 20 times (5 g.) the quantity of albedo, that I found to be effective of flavedo (0·25 g.) with the lemon, as a supply of vitamin B.

In conclusion, my thanks are due to Prof. T. B. Wood, F.R.S. for his kindness in allowing this investigation to be undertaken. I also wish to express my sincere thanks to Mr E. T. Halnan of the School of Agriculture, Cambridge for his advice and assistance. Lastly, I am greatly indebted to Mr Frank Wokes for his preparation of the lemon extract and for his interest and suggestions during the course of the work.

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VIII. A MICRO-METHOD FOR THE ESTIMATION OF THE HYDROGEN ION CONCENTRATION OF CAPILLARY BLOOD.

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(*Received December 10th, 1925.*)

The method is, in principle, essentially that of Hawkins [1923], which in turn was a modification of that of Cullen [1922], using blood instead of plasma. Hawkins collected 0·25 cc. of blood from a vein in a graduated pipette, delivered it into neutralised saline containing indicator under paraffin, centrifuged down the red cells and matched the supernatant fluid against standard phosphates.

Hollo and Weiss [1924] adopted a procedure similar to that of Hawkins but differing in some details.

Our method differs from that of Hawkins in that only a single drop, about 20 mm.^{3}, of blood is required. Further, the tube containing the diluted blood is sealed up, the use of liquid paraffin is avoided and loss of CO_2 cannot occur during the centrifugalisation.

The plasma is diluted 30 times, which, as Cullen [1922] has shown, is sufficient to reduce the influence of the proteins on the indicator used to nearly negligible proportions. This dilution also renders the disturbing effect of the colour of the serum inappreciable. The diluting fluid contains sufficient oxygen to complete the oxygenation of the haemoglobin. Accordingly, the p_H measured is that of the sample when fully oxygenated, without loss of CO_2 and cooled to room temperature. We employ capillary blood which, as Lundsgaard and Moller [1922] and Verzar and Keller [1923] have shown, is 90–95 % saturated with oygen. The method may, therefore, be taken as measuring the p_H of arterial blood cooled down to 18°. The correction to be applied to this measurement to arrive at the p_H of the same blood at the temperature of the body will be discussed later.

Details of the Method.

Requirements.

(1) A freshly prepared saline solution containing 0·7 % NaCl and 0·2 % potassium oxalate or 0·2 % sodium fluoride. Shortly before use, one volume of 0·02 % phenol red is added to 10 volumes of saline and the p_H adjusted to about 7·5 with $N/50$ NaOH containing the same amount of indicator. It is kept in a hard glass bottle protected from CO_2.

(2) A set of standard tubes made of resistance glass, 7 cm. long and 2·5 mm. internal bore. These are filled with $M/15$ phosphate solutions of p_H varying from 7·3 to 7·6 at intervals of 0·05, to which one-tenth volume of 0·02 % phenol red has been added, and sealed off.

The phosphate solutions can be made from appropriate mixtures of $M/15$ NaH_2PO_4 and Na_2HPO_4 obtained from Sörensen's [1912] chart, or Table IV, p. 509, in Cullen's [1922] paper which is derived from Sörensen's data. Another convenient way is to mix a $M/15$ solution of phosphates of p_H 7·5 with one of p_H 6·5 or 10·5 respectively, according to the graph supplied by Dale and Evans [1920]. Their graph is drawn from data calculated from Sörensen's values. These standards must be made up frequently as they fade.

(3) A number of pieces of resistance glass tubing about 8 cm. long and of 2·5 mm. internal bore drawn out at each end to capillary dimensions so as to leave about 7·5 cm. from shoulder to shoulder. The glass tubing must be selected and prepared as described below under "special precautions."

Procedure.

A mark is made on one of the pieces of resistance tube at 0·5 cm. from the shoulder of one end, which we will call A. The tube is then filled from end B up to this mark with the saline containing phenol red and placed ready in a horizontal position. A large drop of blood is obtained from the finger or ear by a deep puncture and the end B of the tube, already filled with saline, is placed in it. End A is slightly depressed and the blood is allowed to flow in, pushing the saline in front of it, until the saline reaches *the beginning* of the capillary at end A. When this has occurred, end B is wiped dry and end A sealed in a peep-flame. As this end cools, blood is sucked into end B which is also sealed either in the flame or with sealing-wax. The blood and saline are at once mixed by rotating the tube between the fingers to avoid clotting. The corpuscles are then separated by spinning in a hand centrifuge for a couple of minutes and the colour of the mixture of saline and plasma compared with that of the phosphate standards.

The matching is done by holding the tubes in a good light at an angle of 45° to a piece of white paper lying on the bench. The tubes should be held by the capillary ends so that they are not warmed by contact with the hand. Greater accuracy is obtained if they are placed in test-tubes of water at 18° containing a slip of white filter paper as background.

If the unknown is found to lie between say 7·35 and 7·4 another standard of p_H 7·375 may be made up and the relation of the unknown to this determined. With a little practice, and p_H around 7·4, it is possible to place the unknown between two standards 0·025 p_H apart. The range of p_H over which such fine adjustment can be made will depend on the colour vision of the individual worker. For most purposes, however, intervals of 0·05 in the p_H of the phosphate standards are close enough. This enables a determination to be made within $\pm$ 0·02 p_H.

Special precautions.

1. The stock phosphate solutions must be made up with the greatest accuracy attainable. We test ours electrometrically when made up and from time to time afterwards. They must be kept in hard glass bottles and protected from evaporation and from CO_2 when withdrawing some by a trap of soda-lime on the inlet tube. It is convenient to have a micro-burette attached to each. The arrangement is that usually employed for standard $Ba(OH)_2$. It is also described by Stitt [1921]. In this case the solutions do not come into contact with laboratory air after they are transferred to the bottles. Even with this precaution, however, watch must be kept upon the more alkaline solution.

2. The indicator should only be added to the saline solution shortly before use and the p_H adjusted to 7·5. The error which may arise from using saline of a p_H differing markedly from that of the blood is indicated in Table I. If the p_H of the blood is found to be abnormally high or abnormally low it is advisable to adjust the p_H of the saline to the level found and make further observations.

Table I. *The effect of the hydrogen ion concentration of the diluting fluid on the p_H found.*

p_H of saline used for dilution	p_H of the serum found
7·2	7·55
	7·56
7·35	7·575
	7·575
7·75	7·625
	7·625

The sample of serum employed was saturated with hydrogen containing about 4 % CO_2 and its p_H electrometrically was 7·59.

3. The whole reliability of the method depends on the small glass tubes not furnishing alkali. Only resistance glasses are suitable and these must be individually tested as described below, as glass from the same batch varies.

Pieces of tubing are washed and left standing in boiled distilled water containing phenol red. If after a few hours the contents of the tubing become pinker, the glass is unserviceable. If no change in colour occurs, the tubing is dried, cut into lengths and the ends drawn out.

As most resistance glass yields alkali temporarily after being melted in the flame, the tubes are rinsed in distilled water and left to soak for some hours in boiled out distilled water containing phenol red. If no pink colour develops inside the tubes they are fit for use. They are rinsed with distilled water and dried with alcohol. *They must not be dried in a hot oven.*

4. In filling the tubes the minimum of air necessary for easy sealing should be left and entrance of CO_2 from the peep-flame during sealing avoided.

If it occurs a fall in the p_H of the saline in the neighbourhood of the seal will be obvious.

5. Unless NaF is used, separation of the corpuscles and comparison with the standards should be undertaken forthwith. With oxalate, the p_H gradually falls from glycolysis, as first shown by Evans [1922]. At room temperature no change can be detected after an hour, but at 38° a fall is discernible after a quarter of an hour. The use of fluoride as anti-coagulant inhibits glycolysis (Evans). If 0·2 % NaF is employed the p_H generally remains constant for 12 hours at 18°. This is a convenience in clinical work, as the sample of blood can be taken and mixed with the saline at the bedside and the remaining stages proceeded with at leisure.

6. The colorimetric observations must be made at or about 18°. The p_H attributed to a particular colour of the indicator in Sörensen's various phosphate mixtures is only valid for 18° so that if the comparison is made at another temperature the result will be incorrect. When using phenol red the error from this cause is of no great moment provided the temperature change does not exceed ± 3°, as this would lead to a difference of only 0·015 in p_H. Greater accuracy is obtained if the standard tubes and the blood-containing tubes are placed in test-tubes of water at 18° as described above, p. 38.

Magnitude of the error due to loss of CO_2 from the blood.

The effect of loss of CO_2 from a small volume of blood might have been expected to invalidate our method, but it is surprising how slowly a drop of blood loses enough CO_2 appreciably to alter its p_H.

We collected some blood from the left ventricle of a rabbit under ether in a syringe which had been rinsed out with 10 % oxalate. The p_H of this blood was determined firstly without any exposure to air by placing the tip of the capillary end of one of our tubes inside the nozzle of the syringe. Subsequently the opportunity for the blood to lose CO_2 was varied in the following manner. Drops of the blood were expressed on to a greasy slide and allowed to remain for different lengths of time before being run into the tubes. The p_H was determined as described above.

After exposure of the blood for 1 minute an alteration in the p_H was not appreciable. After 3 minutes' exposure the p_H had risen 0·05. After 5 minutes the p_H was 0·11 and at 7 minutes 0·17 higher. As was to be expected the effect of loss of CO_2 from a drop of plasma could be detected much sooner. At the end of 1 minute's exposure on a slide the p_H of the plasma had risen 0·06, at the end of 2 minutes 0·09, after 7 minutes 0·19.

As the blood can generally be collected and the tubes sealed off in 30 seconds the error due to loss of CO_2 from the blood in this time is negligible, but if more than a minute is taken the results will be a little too high.

The effect of dilution.

We have found, in agreement with Cullen [1922], that it is immaterial whether the blood is diluted 1/15 or 1/20, so that the rough measurement afforded by the relation of the relative length of the column of blood to the column of saline is sufficient to obviate the protein error of phenol red, provided always that at least a 1/15 dilution is made. Plasma or serum should be diluted 1/30.

The effect of dilution on the p_H found with capillary blood and with a serum equilibrated with a hydrogen CO_2 mixture, the p_H of which was determined electrometrically to be 7·59, is shown in Table II.

Table II.

Fluid examined	Dilution	p_H found
Capillary blood	1/ 5·5	7·4
	1/ 7·5	7·4
	1/14	7·525
	1/15	7·5
Serum saturated with H_2, CO_2 mixture	1/13	7·525
	1/15	7·525
	1/25	7·55
	1/30	7·575
	1/40	7·575

The measure of agreement obtained by repeated observations on the same sample.

A number of consecutive readings of the p_H of oxalated blood collected in a syringe from the left ventricle of an anaesthetised rabbit are shown in Table III. The low p_H is due to the anaesthetic. The first five observations were made by passing the capillary end of the tube inside the tip of the syringe in order to collect the sample of blood. In the last two observations (6 and 7) a drop of blood was pressed to the end of the syringe and exposed to the air during the very short time required for the collection of the sample. Again, it will be seen that the difference between the p_H in these two methods of sampling was within the experimental error of the method.

Table III. *The p_H of arterial blood of a rabbit under ether.*

Number of sample	p_H found	
1	7·24	
2	7·225	
3	7·24	
4	7·21	
5	7·225	Mean of 5 observations 7·227
6	7·235	
7	7·235	Mean of 2 observations 7·235

The results obtained from successive samples of capillary blood from the finger are shown in Table IV. The samples were taken at intervals of 15 minutes and the blood diluted 1/15 with saline of p_H 7·45.

Table IV.

Date of observation	p_H found	
4. vi. 25	7·52	(temp. 22°)
	7·53	
	7·56	
5. vi. 25	7·54	(temp. 21°)
	7·52	
	7·56	
	7·56	
	7·55	

Comparison of the results obtained by the colorimetric method with electrometric measurements.

Evans [1921] came to the conclusion that there was a considerable and fundamental discrepancy between colorimetric and electrometric determinations of the reaction of blood or bicarbonate solutions; and that the former indicated a p_H 0·2 higher than the latter. He was of the opinion that the colorimetric was correct. In view of Evans' observations, Cullen and Hastings [1922] compared carbonate and phosphate solutions equilibrated with definite CO_2 pressures, but found agreement between the results by the two methods. Verney and Bayliss [1923] also failed to find any systematic difference, either with $NaHCO_3$ solution or blood.

Hastings and Sendroy [1924] have recently collected together thirty comparative observations of the p_H of plasma by the electrometric and colorimetric methods, made by Cullen, by Hawkins and by themselves. The average deviation in p_H was only 0·003. We have also made observations on this point and find that there is no serious discrepancy between the two methods either with bicarbonate solutions or plasma provided the concentration of Na^+ is about the same as that in blood plasma.

For the electrometric observations we employed a hydrogen electrode which permitted agitation and deoxygenation of the liquids examined. The other half-cell was a 3·5 *N* KCl calomel electrode. The chain was tested before use with Michaelis' "standard acetate" (0·1 *N* $H\bar{A} + 0{\cdot}1 - Na\bar{A}$) in the hydrogen electrode.

We regard the value 4·626 for the p_H of "standard acetate" at 18° derived from Walpole's [1914] observations to be more nearly correct than the value 4·604 now attributed to it by Michaelis and Kakinuma [1923]. This is, however, a matter of no moment for the present investigation, as the p_H values of the phosphate solutions used for colorimetric comparison were also arrived at on the basis that the p_H of standard acetate is 4·626 and that the E.M.F. against a 3·5 *N* KCl calomel electrode is 0·5218 at 18°. Any small discrepancy between the E.M.F. obtained and 0·5218 was allowed for in the subsequent determinations.

A saturated KCl bridge was employed and it was assumed that diffusion potential was eliminated. The E.M.F. observed was corrected for dry hydrogen at 760 mm. pressure.

As the electrometric determination can only be made on completely reduced blood, whereas the colorimetric is only suitable for oxygenated blood, comparison of the two methods cannot be made with whole blood. The entrance of oxygen into the haemoglobin molecule increases its acid dissociation constant, according to Hastings, Van Slyke and others [1924], about 30 times, according to Brown and Hill [1923] 69 times. This makes a considerable difference to the p_H of blood, if the CO_2 formed cannot get away.

We were, therefore, constrained to use serum or plasma for comparing the two methods. To test our micro-method horse serum was brought into equilibrium with hydrogen containing 3·5 % CO_2 and the p_H determined electrometrically by one of us and colorimetrically by the other, both observations being made at 18°. By the electrometric method the p_H was found to be 7·59, by the colorimetric it was found to be between 7·55 and 7·60 and indistinguishable from 7·575. Another experiment was made with human plasma in equilibrium with hydrogen containing 6·37 % CO_2 at 19°. The p_H arrived at electrometrically was 7·197; duplicated colorimetric observations gave 7·18 and 7·22.

The agreement is good and we conclude that we can determine the p_H of a drop of serum or plasma by our micro-method with a precision not far short of that which we attain with larger quantities electrometrically and we have no reason to suppose that the results with blood would not be equally satisfactory, were we able to make the comparison.

Correction to be applied to colorimetric determinations at 18° *to obtain the* p_H *of the same blood at* 38°.

In the case of plasma, this correction can be ascertained by determining its p_H at 38° electrometrically and, without loss of CO_2, at 18° or 20° colorimetrically. Cullen [1922] did this and found that, on the average, the p_H of human plasma was 0·22 and of horse serum 0·16 higher at 20° than at 38°. We have repeated Cullen's experiment with a sample of horse serum, using the micro-method, and obtained 0·18 rise for 20° fall in temperature.

Cullen's results showed rather wide variations when using the sera of different species of animal but, as the effect of temperature will vary with the relative concentration of CO_2, $NaHCO_3$ and proteins in the particular serum, this is not surprising.

Cullen's experiments, however, fail to decide what is the appropriate correction to apply to observations of the p_H of plasma at room temperature to obtain the p_H of the corresponding blood at 38°. Nor does the manœuvre of Hastings and Sendroy [1924], who determine the p_H of diluted plasma at 38° and allow for the increased dissociation of the indicator, succeed in determining this. In all these experiments the corpuscles were not separated from the plasma at body temperature, so that a "true plasma" was not investigated.

Hasselbalch [1917] found the effect of temperature on the p_H of blood

and serum, at constant CO_2 pressure, to be very different. It would be unsafe, therefore, and in view of the high value for the heat of ionisation of haemoglobin found by Brown and Hill [1923], to assume, without further enquiry, that when the total CO_2, bound and free, remains constant the effect of changing the temperature from 18° to 38° is the same with blood as it is with plasma. In a following paper we shall deal with the effect of temperature upon the p_H of plasma and blood respectively. So far, our results indicate that the p_H of both is very similarly affected by change in temperature, when the total CO_2 in the system remains constant. Accordingly, if Cullen's correction of − 0·2 be applied to the p_H of human blood determined colorimetrically at 18°–20° the result will closely approximate to the p_H of the arterial blood at body temperature.

SUMMARY.

A method by which the p_H at room temperature of a drop of capillary blood can be ascertained with an error of ± 0·03 is described.

The correction necessary to convert this to the p_H of the blood at 38° is discussed.

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IX. THE DISCREPANCY BETWEEN ELECTROMETRIC AND COLORIMETRIC (PHENOL RED) DETERMINATIONS OF c_H ACCORDING TO THE SALT-CONTENT OF THE SOLUTION.

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The method of Friedenthal [1904] and Salm [1904] for the approximate determination of c_H by adding various indicators to the solution was developed by Sörensen [1909] into a colorimetric method of considerable precision and great usefulness for determining p_H and is now universally employed.

Michaelis and Rona [1908, 1909] were the first to draw attention to aberrations of the colorimetric method due to the salt-content of the solution under investigation. For accurate work it is necessary to be aware of these and to correct for them. The entire blame was attributed to the indicator, until it was pointed out by Sörensen and Palitzsch [1910] that the addition of NaCl or KCl to a buffer solution of phosphates also altered the c_H, as determined electrometrically, and that the discrepancy between the two methods was the algebraic sum of the influence of the electrolyte upon the dissociation of the weak acid of the buffer mixture and the indicator respectively.

The earlier literature will be found in Sörensen's article on the measurement and significance of hydrogen ion concentration [1912].

For the purpose of their investigation into the p_H of sea-water, Sörensen and Palitzsch [1913, 1, 2] determined the discrepancy occurring with concentrations of NaCl up to 4 %, when phenolphthalein and naphtholphthalein were the indicators and the buffer mixture was a "weak" solution of phosphates. From their observations they plotted "error" against NaCl content. The standards for comparison contained $M/15$ phosphate. The colorimetric and electrometric methods agreed when about 0·7 % NaCl was added. The discrepancy without added NaCl and with 4 % salt was about 0·2 in the p_H but in opposite directions. When neutral red was used as indicator the discrepancy was within the error of observation until the concentration of NaCl exceeded 1 %.

Palitzsch [1911] had previously made similar observations on the effect of NaCl when the indicator used was methyl red. His observations are however difficult to interpret, as citrates were used in the tubes for comparison. The tint of this indicator is not quite the same with citrates as with phosphates.

Bjerrum [1914] when discussing the theory of titration with indicators, referred to their "salt error" and concluded that it must be attributed either (1) to a molecular combination of the indicator with the salt whereby its colour is changed, or (2) to the effect of the electrolyte upon the dissociation constant of the indicator.

Michaelis and Gyemant [1920] determined the discrepancy between colorimetric and electrometric observations caused by 0·5 *M* solutions of NaCl and KCl when the nitrophenols and phenolphthalein were used as indicators. The discrepancy caused by certain concentrations of $CaCl_2$, K_2SO_4 and $AlCl_3$ can also be derived from the data in some other of the tables. They discuss the theory of "salt error" at some length.

Kolthoff [1922] published useful observations on the "salt error" of a number of indicators arrived at from electrometric and colorimetric measurements of buffer solutions, generally 0·05 *M* phosphates, with and without the addition of 0·5 *M* NaCl.

Most of the observations recorded above have been made with phosphate buffers, but sometimes borates, acetates or cacodylates were employed. Evans [1921], using a buffer of $NaHCO_3$ in equilibrium with CO_2 pressures, came to the conclusion that in the presence of 0·85 % NaCl there was a constant discrepancy of about 0·2 p_H, the colorimetric method indicating the higher p_H. This he attributed to a fundamental defect of the hydrogen electrode in the presence of bicarbonates. He believed this was due to the reduction of carbonic acid to formic acid at the electrode.

We have used mixtures of sodium phosphates and sodium bicarbonate at constant CO_2 pressure as buffers. The concentration of the buffering salts was varied. The indicator we employed was phenol red. As information regarding the effect of NaCl is more particularly useful for physiological experiments, the variation in the concentration of electrolytes was produced by addition of this salt.

A number of solutions of the buffer containing NaCl in concentrations varying from 0·001 to 1·0 *M* were prepared and the p_H determined electrometrically and colorimetrically. The results are expressed in Tables I to V. From them graphs were drawn with the p_H plotted against the $-\log$ of the concentration of total Na. The figures for the discrepancy between electrometric and colorimetric observations given in the tables are derived from the smoothed curves in the corresponding graphs. The scale of all the graphs is the same.

Methods.

Equilibration of the bicarbonate solutions with constant CO_2 pressure.

A supply of H_2 with a constant percentage of CO_2 was obtained in the following way. A small steel gas-cylinder was washed out with H_2. It was then charged with CO_2 until the pressure was, say, 5 atmospheres. Without undoing any of the connections, which had been previously washed out with H_2, H_2 was run in from a large cylinder until the pressure was 100 atmospheres.

Some of the CO_2 disappeared rather quickly from the first cylinder filled. At the partial pressure of about 5 atmospheres it attacked the steel, forming $FeCO_3$ on the walls, but after the cylinder had been used once, the loss of CO_2 became so slow as to be unimportant. The disappearance of CO_2 from our cylinder is now so gradual that, over a period of one week, it does not exceed the error of analysis.

In our earlier experiments, equilibration was accomplished in a hard glass round-bottomed flask of about 250 cc. capacity. 20 cc. of the solution of bicarbonate was swirled round the wall of the flask mechanically, whilst a mixture of CO_2 and H_2 of known composition, previously saturated with H_2O at 18°, streamed through the flask. It was found that half an hour was sufficient to attain equilibrium. The temperature of the room was maintained at 18° ± 0·5°. At the end of an hour, samples were withdrawn from the flask into the hydrogen electrode and also into the pots of the colorimeter, avoiding contact with air during the transfer.

In the later experiments the indicator was added to the solution before equilibration with CO_2. This was carried out in a rotating bottle inside the thermostat containing the electrode vessels. It was previously ascertained that indicator, in the amount added, did not interfere with the electrometric determinations.

Electrometric measurements.

In the earlier series of experiments (Tables I, II and IV) the hydrogen electrode vessel used was of the U-tube type formerly employed by Michaelis. This was subsequently abandoned for a modification we devised of that used by Walpole [1913] for electrometric titration. Walpole's electrode vessel has an all-glass syringe attached to the H_2 inlet tube by means of which the liquid can be drawn up over the electrode or displaced at will. The handle of the syringe was outside the thermostat so it was not necessary to open the thermostat during the observations.

Our electrodes contained about 4 cc. of liquid. They permitted complete de-oxygenation of the liquid and thorough washing of the electrode with it. By efficient agitation, false equilibria were avoided and once the electrode was charged with hydrogen the final reading was obtained in a few minutes. We found agitation of the liquid and frequent washing of the electrode to be essential when the buffer consisted of bicarbonates and CO_2. The hydrogen

CO_2 mixture supplied to the electrode in this case was the same as that with which the equilibrium with CO_2 was obtained. It was previously saturated with H_2O at 18°.

A 3·5 *N* KCl calomel electrode was used as the other half cell. For the π_0 value of this electrode at 18° 0·2548 volt [Sörensen, 1924] was used. It was tested before each day's observations against "standard acetate" (0·1 *N* $H\overline{A}$ + 0·1 *M* $Na\overline{A}$) and any small discrepancy between the E.M.F. found and 5·218 volts was allowed for in calculating the p_H. The values assigned to the electrometric measurements are those calculated for dry H_2 at 760 mm. pressure.

Colorimetric measurements.

In all but the last series of experiments, the colorimetric determinations were made by the method used by Dale and Evans [1920] for determining the p_H of dialysates from blood. We used a large colorimeter box into which we could get our heads, instead of the small one figured in their paper. The solutions were run into the colorimeter pots under paraffin and precautions were taken to avoid loss or gain of CO_2 during the transfer. The method proved satisfactory when the buffer was a solution of phosphates, but when bicarbonate and CO_2 were used the discrepancy between experiments was more than we liked, sometimes amounting to 0·06 p_H. Therefore, many observations had to be made and the averages taken (see Table IV).

In the series of experiments detailed in Table V the technique was modified as follows. The indicator was added to the solutions of bicarbonate before they were brought into equilibrium with the mixture of H_2 and CO_2. When equilibrium had been obtained the solution was drawn into the electrode vessel without contact with air and the E.M.F. measured. This being accomplished, samples were withdrawn *from the electrode vessel* into small hard glass tubes drawn out at each end, and the ends immediately sealed in a flame. The sampling tubes were filled with the mixture of H_2 and CO_2 before the liquid from the electrode was allowed to flow into them. Subsequently, the apparent p_H of the solution was determined by matching the colour against freshly made standard solutions of *M*/15 phosphates containing the same amount of indicator in similar tubes. With the adoption of this technique, our troubles disappeared and duplicate observations agreed.

THE EFFECT OF DILUTION ON THE p_H OF PHOSPHATE SOLUTIONS AS DETERMINED ELECTROMETRICALLY AND COLORIMETRICALLY.

Diluting a phosphate mixture considerably increases its p_H. As may be seen from Table I or Fig. 1 the p_H increases 0·47 in going from 0·3 *M* to 0·001 *M*. Michaelis and Kruger [1921] pointed out this fact, but it is not generally appreciated. Under the same conditions the apparent p_H from colorimetric measurements changes much less. At a concentration of *M*/15 the electrometric and colorimetric estimations agree because this is the

ERRATUM

Page 49, Table I at head of first column:

for Dilution NaCl *read* Dilution PO_4

concentration of phosphates in the standards with which they are compared. Dilution of the phosphates affects the dissociation of the indicator as well as the c_H of the solution or there would be no discrepancy. The former effect to some extent compensates the latter. Consequently, when the electrometric and colorimetric observations are plotted against the concentration of phosphates in solution, as in Fig. 1, the curves cross at $M/15$. If the colour comparison had been made with standards containing $M/1000$ phosphates instead

Table I. *Effect of dilution on the* c_H *of a phosphate mixture* (8·5 *parts* Na_2HPO_4 + 1·5 *parts* NaH_2PO_4) *when determined electrometrically and colorimetrically with phenol red. Temperature* 18°.

(The standards for colorimetric comparison contained $M/15$ phosphates.)

Dilution NaCl	Dilution Na	p_H electrometric	p_H colorimetric*	Discrepancy (from smoothed curve)
$M/3$	$M/$ 1·62	7·36	7·44	+0·07
$M/6$	$M/$ 3·24	7·45	7·48	+0·04
$M/12$	$M/$ 6·48	—	7·53	+0·01
$M/15$	$M/$ 8·1	7·55	7·55	0
$M/25$	$M/13·5$	—	7·57	−0·02
$M/30$	$M/16·2$	—	7·58	−0·03
$M/40$	$M/21·6$	7·65	7·61	−0·045
$M/100$	$M/54$	—	7·64	−0·09
$M/150$	$M/81$	7·74	7·66	−0·11
$M/225$	$M/102$	7·78	—	—
$M/400$	$M/216$	7·81	7·66	−0·15
$M/1000$	$M/540$	7·83	7·65	−0·16

When the figures under "discrepancy" have a + sign it signifies that the colorimetric is higher than the electrometric.

* The dilution of the solutions for the colorimetric determinations was 10 % greater than that for the electrometric, due to solution of indicator added.

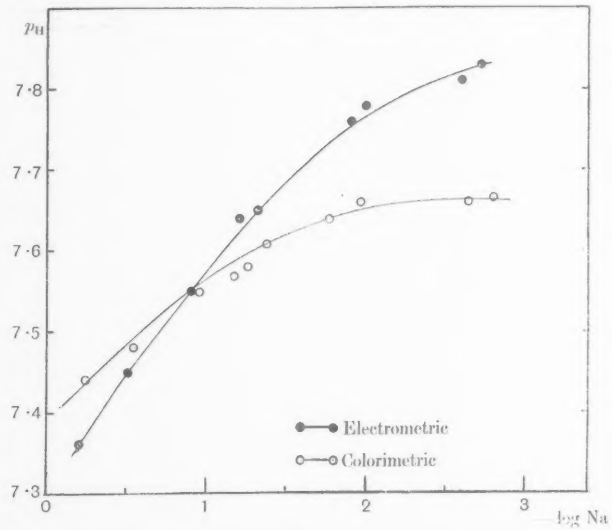

Fig. 1.

of $M/15$ the two curves would agree at this point and not cross, but the colorimetric curve would fall less rapidly than the electrometric as the concentration of phosphates increases.

In all the graphs p_H is plotted as ordinate against the logarithm of the dilution of Na as abscissa, *i.e.* the negative logarithm of c_H is plotted against the negative logarithm of c_{Na}. This method was chosen so that all the graphs should be comparable. It is also more instructive, for, phenol red having a coloured anion, it is more particularly the concentration of kations which modifies its dissociation. If 0·25 be added to any value along the abscissa it will be equal to the negative logarithm of the concentration of the phosphates.

EFFECT OF NaCl ON THE p_H OF PHOSPHATE SOLUTIONS AS DETERMINED ELECTROMETRICALLY AND COLORIMETRICALLY. (Tables II and III and graphs in Figs. 2 and 3.)

We used 0·0044 and 0·01 M solutions of phosphates. The concentration of NaCl varied between 0·001 and 1·0 M. The effect upon the p_H was greater with the less buffered solutions (Table II and Fig. 2). This fact was noticed by Palitzsch [1911]. The influence of increasing the concentration of Na^+ by adding NaCl is more pronounced than when it is increased by augmenting the concentration of the phosphate mixture as in the experiment detailed in Table I and illustrated in Fig. 1. This is presumably because in Exp. 1 the concentration of the buffer is increased *pari passu* with the increase in concentration of kations.

The effect of increasing the salt concentration on the p_H of the solution is similar to that found by Michaelis and Kruger [1921].

In both Exps. 2 and 3 the colorimetric estimation coincides with the electrometric when the concentration of kations is about equal to that of the $M/15$ phosphate solution used for making the colorimetric comparisons.

Table II. *Effect of NaCl on the* c_H *of a* 0·0044 M *solution of phosphates* (Na_2HPO_4 8·4 *parts*, NaH_2PO_4 1·6 *parts*) *of concentration* 0·0044 M *when determined electrometrically and colorimetrically with phenol red. Temperature* 18°.

(The standards for colorimetric comparison contained $M/15$ phosphates.)

Dilution NaCl	Dilution Na	p_H electrometric	p_H colorimetric*	Discrepancy (from smoothed curve)
$M/1$	$M/$ 0·99	6·82	7·22	+0·36
$M/2$	$M/$ 1·93	7·13	7·37	+0·24
$M/4$	$M/$ 3·88	7·32	7·49	+0·10
$M/8$	$M/$ 7·52	7·53	7·53	0
$M/16$	$M/$ 14·08	7·61	7·56	−0·06
$M/32$	$M/$ 25·64	7·70	7·58	−0·10
$M/100$	$M/$ 55·56	7·74	7·61	−0·14
$M/1000$	$M/111·1$	7·76	7·62	−0·14

When the figures under "discrepancy" have a + sign it signifies that the colorimetric is higher than the electrometric.

* The dilution of the solutions for the colorimetric determinations was 10 % greater than that for the electrometric, due to solution of indicator added.

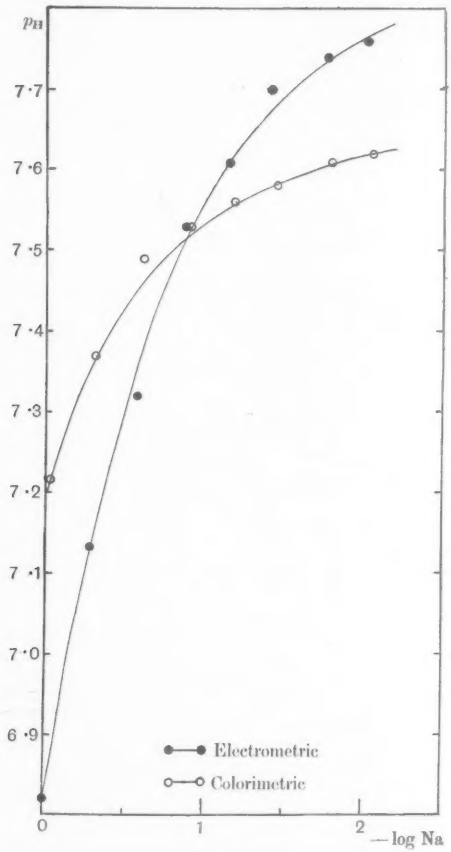

Fig. 2.

Table III. *Effect of NaCl on the* c_H *of a* 0·01 *M solution of phosphates* (Na_2HPO_4 7·75 *parts*, NaH_2PO_4 2·25 *parts*) *when determined electrometrically and colorimetrically with phenol red. Temperature* 18°.

(The standards for colorimetric comparison contained *M*/15 phosphates.)

Dilution NaCl	Dilution Na	p_H electrometric	p_H colorimetric*	Discrepancy (from smoothed curve)
M/1	*M*/ 0·98	6·75	6·98	+0·20
M/2	*M*/ 1·93	6·95	7·10	+0·14
M/4	*M*/ 3·7	7·10	7·20	+0·07
M/8	*M*/ 7	7·215	7·22	+0·01
M/16	*M*/12·4	7·31	7·27	−0·04
M/32	*M*/20·3	7·38	7·31	−0·08
M/128	*M*/38·7	7·45	7·33	−0·12
M/1000	*M*/52·6	7·50	7·36	−0·15
M/∞	*M*/55·5	7·505	7·36	−0·15

When the figures under "discrepancy" have a + sign it signifies that the colorimetric is higher than the electrometric.

* The total Na in the colorimetric observations was 10/11 of that present in the electrometric.

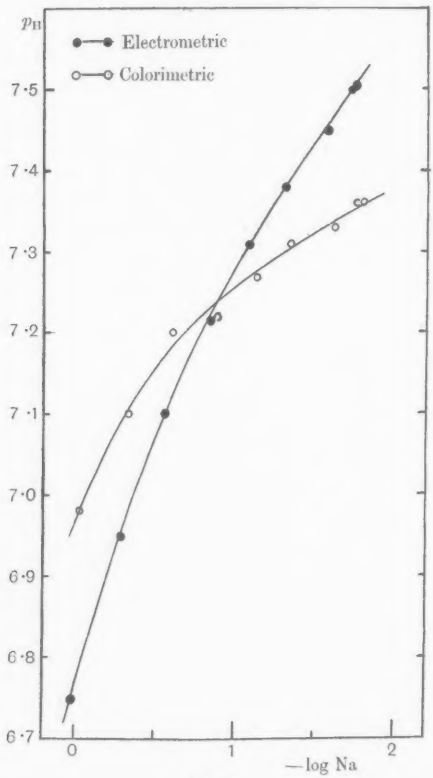

Fig. 3.

EFFECT OF NaCl ON THE p_H OF SOLUTIONS BUFFERED WITH $NaHCO_3$ IN EQUILIBRIUM WITH A CONSTANT PRESSURE OF CO_2. (Tables IV and V, Figs. 4 and 5.)

0·02 and 0·04 *M* solutions of $NaHCO_3$ were used. These were brought into equilibrium with H_2 containing 3·17 % and 5·85 % CO_2 respectively. In the first series of experiments our colorimetric observations of p_H varied as much as 0·05 in duplicate experiments so we had to do a good many and take the means. This involved another error as the barometer was not the same over the period. The maximum error in p_H from this cause was, however, not more than 0·02. In the second experiment as explained above our technique was improved and the observations were all carried out in two days, during which time the barometer varied only a few millimetres.

The graphs on the left hand of Figs. 4 and 5 represent the observations as made. The effect of the addition of electrolyte on the colorimetric p_H has

in these experiments more than compensated for the actual fall in p_H. Before they can be compared with the previous experiments both curves should, however, be made steeper, for, although the CO_2 pressure was constant

Table IV. *Effect of NaCl on the c_H of a 0·02 M solution of $NaHCO_3$ in equilibrium with CO_2 at 24·1 mm. pressure, when determined electrometrically and colorimetrically with phenol red. Temperature* 18°.

(The standards for colorimetric comparison contained $M/15$ phosphates.)

		p_H electrometric			p_H colorimetric*			
Dilution NaCl	Dilution Na	Observations	Mean	Corrected for CO_2 solubility	Observations	Mean	Corrected for CO_2 solubility	Discrepancy (from smoothed curve)
$M/1$	$M/0·99$	7·225 7·23 7·23	7·23	7·10	7·44 7·43 7·42	7·43	7·30	+0·2
$M/2$	$M/1·9$	7·33 7·315	7·32	7·24	7·43 7·44 7·45	7·44	7·36	+0·1
$M/3·7$	$M/3·36$	7·39	7·39	7·35	7·42 7·44	7·43	7·39	+0·04
$M/6·9$	$M/6·1$	7·43 7·40	7·415	7·39	7·43 7·38	7·41	7·40	0·0
$M/16·6$	$M/10·35$	7·44 7·44	7·44	7·44	7·35 7·41	7·38	7·38	−0·05
$M/50$	$M/25$	7·47 7·475 7·48	7·475	7·475	7·36 7·39 7·39	7·38	7·38	−0·11
$M/1000$	$M/47·6$	7·50 7·49	7·495	7·495	7·39 7·36 7·38 7·37 7·36	7·37	7·37	−0·12

When the figures under "discrepancy" have a + sign it signifies that the colorimetric is higher than the electrometric.

* The dilution of the solutions for the colorimetric determinations was 10 % greater than that for the electrometric.

Table V. *The effect of NaCl on the c_H of a 0·04 M solution of $NaHCO_3$ in equilibrium with CO_2 at 44·7 mm. pressure, when determined electrometrically and colorimetrically with phenol red. Temperature* 18°.

(The standards for colorimetric comparison contained $M/15$ phosphates.)

		p_H electrometric		p_H colorimetric		
Dilution NaCl	Dilution Na	Observed	Corrected for CO_2 solubility	Observed	Corrected for CO_2 solubility	Discrepancy (from smoothed curve)
$M/1$	$M/0·96$	7·267	7·142	7·42	7·295	+0·15
$M/2$	$M/1·85$	7·33	7·251	7·445	7·365	+0·11
$M/4$	$M/3·45$	7·38	7·34	7·437	7·397	+0·06
$M/10$	$M/7·14$	7·437	7·417	7·432	7·412	0·00
$M/30$	$M/13·7$	7·473	7·463	7·427	7·417	−0·04
	$M/25$	7·507	—	7·41	—	−0·09

The bicarbonate solution contained the required amount of phenol red.

When the figures under "discrepancy" have a + sign it signifies that the colorimetric is higher than the electrometric.

throughout the observations, the concentration of CO_2 in the solutions varied owing to the diminishing solubility of this gas as the salt in the solution increased. In the graphs on the right-hand side of the figures the observations have been corrected for this by calculating by Hasselbalch's formula what effect the diminished solubility of CO_2 would make upon the p_H and subtracting it.

For these calculations Bohr's [1899] data for the solubility of CO_2 in NaCl solutions of different strengths were used.

It will be seen from Tables IV and V and Figs. 4 and 5 that, even after correcting for the solubility of CO_2, the influence of NaCl upon the p_H of

Table VI. *The effect of NaCl on the* c_H *of phosphate mixtures containing* KH_2PO_4 1·6 *parts and* Na_2HPO_4 8·4 *parts in molecular concentration* (*a*) *M*/15, (*b*) *M*/100.

Phosphate	NaCl concentration molecular	Total Na concentration	p_H
M/100	0	0·0184 *N*	7·743
	0·1	0·1184 ,,	7·49
	0·333	0·3515 ,,	7·28
	1	1·0184 ,,	6·93
	—	—	6·94
M/15	0	0·1214 ,,	7·533
	0·1	0·221 ,,	7·433
	0·333	0·454 ,,	7·26
	1	1·1214 ,,	6·969

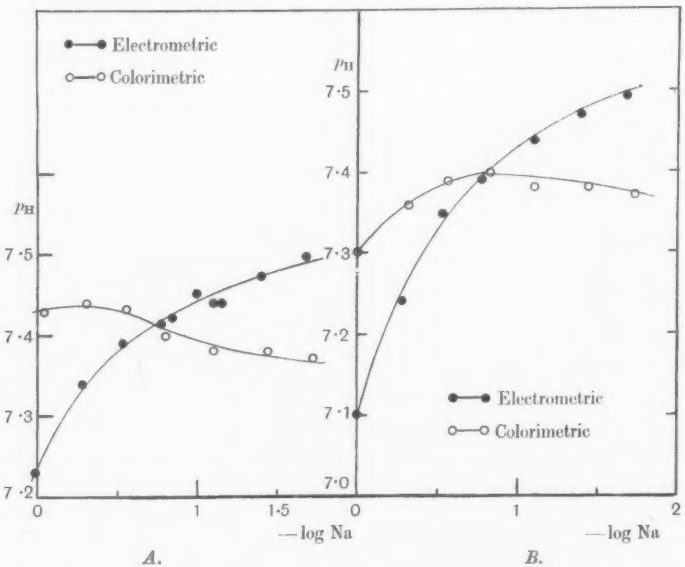

Fig. 4.
$B = A$ corrected for varying CO_2 concentration.

0·02 and 0·04 *M* $NaHCO_3$ solutions is much less than upon 0·01 *M* phosphate solution. The smaller molecular strength of the phosphate buffer is not enough to account for this. Nearly the same drop in p_H is produced by NaCl upon 0·067 *M* as upon 0·01 *M* phosphate solution, as shown in Table VI. The curves cross at a point where the $-\log$ of the concentration of the Na is $0{\cdot}85 = 0{\cdot}142\ N$ Na which is about the concentration in 0·85 % NaCl or serum. At this point there was no discrepancy between the colorimetric and electrometric estimations.

The curves drawn from experiments in which the buffer was a phosphate mixture (Figs. 1, 2 and 3) appeared to intersect when the concentration of sodium was a little less, viz. 0·123 *N*. We are uncertain whether there is a real difference in the concentration of Na^+ at the point of intersection in the two cases. A small error in drawing the curve or one of 0·02 p_H in the observations would be more than sufficient to account for this.

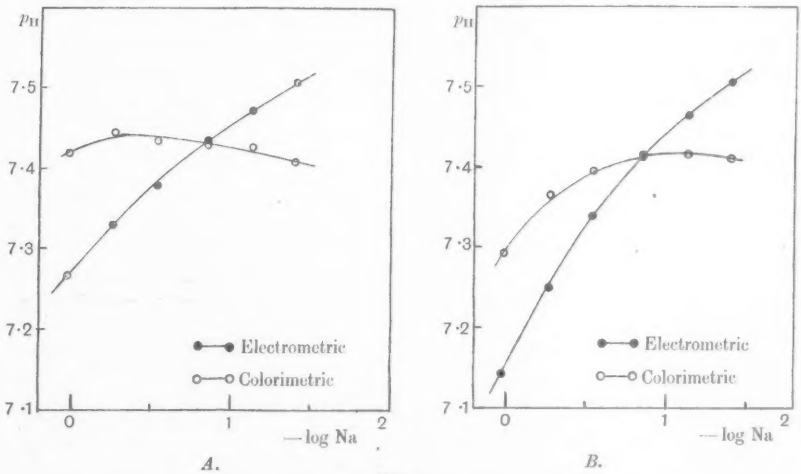

Fig. 5.
B = *A* corrected for varying CO_2 concentration.

Discussion.

We have found the data derived from the experiments described above of service for correcting our colorimetric observations with phenol red and may be they will be useful to others. Unfortunately, however, these corrections for NaCl content with phosphate or carbonate buffers cannot be applied if another indicator is used, or in the case of other buffers or if other electrolytes are present in considerable concentration. Even with phosphate and carbonate buffers they are only valid for phenol red and only accurate for the concentrations of buffer stated.

From the observations of Michaelis and Kakinuma [1923] the effect of NaCl upon the p_H of a mixture of 0·01 *M* $H\overline{A}$, and 0·01 *M* $Na\overline{A}$ is considerably less than Michaelis and Kruger [1921] found upon phosphates "much diluted." They made the interesting discovery that the influence of the kations of the univalent alkali metals upon both phosphate and acetate solutions varies in the inverse order of their atomic weights. Bivalent kations were more potent. It is probable that, similarly, the effect of different kations upon the dissociation of indicators will be found to vary. At present, therefore, it seems to be necessary to ascertain the discrepancy of the colorimetric method for the particular conditions under which one is working.

As far as the influence of NaCl on phosphate and bicarbonate buffers is concerned, it is clear, from our experiments, that with phenol red coincidence of colorimetric with electrometric observation only occurs when the concentration of Na^+ is about the same as that of the phosphate solutions used for colorimetric comparison. If it is more, the colorimetric gives too high results, if less, too low results.

Our observations with bicarbonate buffers lend no support to Evans' [1921] conclusion that the hydrogen electrode is incapable of affording the correct E.M.F. in solutions of 0·02 *M* bicarbonate. We think the systematic discrepancy of 0·2 p_H found by him between the electrometric and colorimetric p_H of 0·2 *M* $NaHCO_3$ dissolved in 0·18 *M* NaCl must be explained in some other way. According to our experiments (Table IV, Fig. 4 and Table V, Fig. 5) the p_H of 0·02 *M* $NaHCO_3$ in 0·18 *M* NaCl solution, determined by the two methods, would agree within the error of experiment.

Cullen and Hastings [1922] made electrometric and colorimetric observations of the p_H of 0·06 *M* $NaHCO_3$ solution and three mixtures of $NaHCO_3$ and phosphates. The solutions were equilibrated with CO_2 at constant pressure. Phenol red was the indicator used and Sörensen's phosphate solutions were the standards for colorimetric comparison.

They found good agreement notwithstanding that the total kations in the solutions varied from 0·06 *M* to 0·13 *M*. In view of our experiments detailed above, we should not expect such coincidence except in their third experiment in which the concentration of Na was 0·13 *M*. The conditions of Cullen and Hastings' experiment were, however, different from ours. Their solutions were strongly buffered and therefore not so sensitive to concentration of kations. Moreover, the variation in kation concentration between the different solutions was brought about by altering the amount of buffer, which tends to obliterate the effect.

Verney and Bayliss [1923] have also compared electrometric and colorimetric measurements of the p_H of 0·02 *M* $NaHCO_3$ in equilibrium with different pressures of CO_2. The indicators used were phenol red, cresol red and neutral red. They satisfied themselves that there was no systematic difference between the results by the two methods. We do not understand how they found agreement when phenol red was the indicator used and the

colorimetric standard contained $M/15$ phosphates, unless they dissolved the bicarbonate in 0·85 % NaCl. According to the observations in Table IV and Fig. 4, the colorimetric gives results 0·1 p_H too low with 0·02 M $NaHCO_3$ in water. It is evidently difficult for different observers to attain concordant results.

Our observations explain why, as found by Cullen [1922] and confirmed by us [1925], serum diluted 1 in 30 with 0·85 % NaCl is found to have the same p_H by the colorimetric as that obtained by the electrometric method in the undiluted serum, for, notwithstanding the dilution, the colorimetric comparison is made in a solution containing approximately the same concentration of Na^+ as the standard phosphate solution used for comparison.

As to the cause of the fall in the p_H of the solutions and the increased dissociation of the phenol red produced by NaCl we have little to contribute. In the light of Bjerrum's "activity" theory, Michaelis and Gyemant [1920] attribute the effects to diminished activity of the anions both of the buffer salt and the indicator, due to the added kations. This theory interprets the fundamental facts by simple reference to the mass law. It is also capable of explaining an old observation of Arrhenius [1899] that the hydrolytic action of acetic and formic acids was increased by the addition of KCl or NaCl. But there are other facts, such as the increase in c_H of $M/500$ HCl [Michaelis and Kakinuma, 1923; Sörensen, 1924] when salt is added which cannot be explained so simply. For the explanation of these it seems necessary to assume, as Arrhenius did, that salt increases the dielectric constant of water.

Our observations show that the fall in p_H produced by a given concentration of kations depends upon the nature of the buffer, and that the quantitative relationship between the electrolyte added and the concentration of the buffer salt also influences the result. Why the discrepancy between the colorimetric and electrometric values should be greater, the weaker the buffer solution, is not clear, for the concentration of indicator remains constant throughout.

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X. THE OXIDATION OF ACETOACETIC ACID AND ESTER BY HYDROGEN PEROXIDE AND ITS BIOCHEMICAL SIGNIFICANCE.

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It is commonly believed at the present time that acetoacetic acid, when it appears as an intermediate product of animal metabolism, is produced by the incomplete oxidation of fatty acids or certain of the amino-acids. Further, it is only produced under conditions in which the oxidation of carbohydrates is in abeyance. For this reason it has been held that the complete oxidation of acetoacetic acid can only occur when carbohydrate is being simultaneously oxidised and that at this stage some form of combination between acetoacetic acid (or its reduction product, β-hydroxybutyric acid) and a carbohydrate is essential [Geelmuyden, 1904; Ringer, 1914; Woodyatt, 1916]. Shaffer [1921] has produced an *in vitro* analogy to this biological process. He finds that acetoacetic acid is more rapidly oxidised by hydrogen peroxide in the presence of glucose and other carbohydrates than in their absence, but, so far as we are aware, no explanation of the mechanism of this reaction has been given. The same facilitation of the oxidation of acetoacetic acid is produced however by the presence of other substances, not carbohydrates [Ernst and Forster, 1924], so that as an *in vitro* analogy the reaction is less satisfactory than Shaffer at first believed. Owing to the lack of knowledge as to the products produced when acetoacetic acid and other β-keto-acids undergo oxidation, we have thought it advisable to study the oxidation of acetoacetic acid by hydrogen peroxide in the hope of finding some indication of the mechanism of the reaction and thus, possibly, of the method by which it may be oxidised in the body.

We are well aware that results of experiments of this kind can only be applied to the living animal with considerable reserve, but they may often lead to a more fruitful investigation of biological processes of oxidation than would be possible without them.

There is little previous work on the subject. In an unpublished observation referred to in his book, Dakin [1924] states that sodium acetoacetate when oxidised with hydrogen peroxide yields acetic, glyoxylic and formic acids and carbon dioxide. Engfeldt [1921], oxidising sodium acetoacetate

with potassium permanganate, states that the products are probably acetic and glyoxylic acids, but that most of the glyoxylic acid is further oxidised to oxalic acid. The possibility that intermediate oxidation products might be formed other than those just mentioned was realised in our earliest experiments. It was found that an aqueous solution of the sodium compound of acetoacetic ester, or of the acid, is readily oxidised by hydrogen peroxide in the cold, the reaction in fact causing a considerable rise of temperature. When the reaction is over a substance is present which reduces Fehling's solution strongly in the cold and is therefore not glyoxylic acid. If the products of the reaction are distilled under reduced pressure, the aqueous distillate also reduces Fehling's solution in the cold and gives an osazone which indicates that the reducing substance found in the distillate is either methylglyoxal or monohydroxyacetone (acetol). Evidence collected subsequently proved that this substance is monohydroxyacetone. Since the mechanism of its production was not clear (either α- or γ- oxidation would account for its presence, thus:

$$CH_3 . CO . CH_2 . COOH \rightarrow CH_3 . CO . CH(OH) . COOH \rightarrow CH_3 . CO . CH_2(OH) + CO_2$$

or

$$CH_3 . CO . CH_2 . COOH \rightarrow CH_2(OH) . CO . CH_2 . COOH \rightarrow CH_2OH . CO . CH_3 + CO_2),$$

a careful examination by various methods of the solution resulting from the oxidation was undertaken. In this way a method was gradually evolved by which a considerable number of substances could be separated from the oxidation mixture, and this is described in the Experimental Section. The oxidations were carried out in a freezing mixture. The rapid rise of temperature and disappearance of the added peroxide ceased after the addition of about two-thirds of a molecule of peroxide per molecule of acetoacetic ester. When the sodium salt of the acid is used instead of the ester the oxidation is not so rapid but appears to pursue the same course. Almost the whole of the work has been done with the sodium compound of the ester because of certain advantages which it offered in the subsequent separation of the products. The substances isolated, or separated, in the form of derivatives from the solution after oxidation by not more than one molecular proportion of peroxide in the cold are: a little unchanged acetoacetic ester and acid, ethyl acetate, ethyl isocarbopyrotritartrate, acetonylacetoacetic acid and ester, acetonylacetone, acetol and a small quantity of acetic, glyoxylic and oxalic acids. In addition, a strongly reducing but very unstable acid is present which could not be isolated in a pure state owing to its instability. It may be regarded as either α-hydroxyacetoacetic acid or $\alpha\beta$-dihydroxycrotonic acid. These substances, together with carbon dioxide and a certain amount of acetone and alcohol produced by hydrolysis of the acetoacetic ester, account practically quantitatively, so far as can be determined by isolation methods, for the whole of the products of the reaction. The primary oxidation product appears to be the unstable reducing acid. This

probably arises by the addition of hydrogen peroxide or oxygen to the enolic form of acetoacetic acid at the double bond,

$$\left.\begin{array}{l} CH_3 . C(OH)=CH . COOH \\ \quad + HO \ldots OH \\ \text{or} \quad + O \end{array}\right\} \rightarrow \left\{\begin{array}{l} CH_3 . CO . CH(OH) . COOH \\ \text{or} \\ CH_3 . C(OH)=C(OH) . COOH \\ \text{or} \\ CH_3 . \underset{\llcorner\!\!-O-\!\!\lrcorner}{C(OH)} . CH . COOH \end{array}\right.$$

the compound produced subsequently decomposing to give acetol and carbon dioxide;

$$CH_3 . CO . CH(OH) . COOH \rightarrow CH_3 . CO . CH_2(OH) + CO_2.$$

That this oxidation product is $\alpha\beta$-dihydroxycrotonic acid or one of its isomeric forms, *e.g.* α-hydroxyacetoacetic acid, appears to be confirmed by the fact that oxidation of $\alpha\beta$-dihydroxybutyric acid by means of Fenton's reagent gives a similar acid which reduces Fehling's solution rapidly in the cold and gives immediately the dinitrophenylhydrazone of methylglyoxal on treatment with nitrophenylhydrazine.

Of the remaining products, acetonylacetoacetic ester and acid are found in considerable amounts. These may have arisen in two ways. Either a secondary oxidation occurs involving two molecules of acetoacetic ester, yielding diacetylsuccinic ester which after hydrolysis loses carbon dioxide to give acetonylacetoacetic acid (I),

$$\begin{array}{l} CH_3 . C(OH)=CH . COOC_2H_5 \\ CH_3 . C(OH)=CH . COOC_2H_5 \end{array} \xrightarrow{+O} \begin{array}{l} CH_3 . C(OH)=C . COOC_2H_5 \\ CH_3 . C(OH)=C . COOC_2H_5 \\ CH_3 . C(OH)=CH \\ CH_3 . C(OH)=C . COOH \end{array} \rightarrow \begin{array}{l} CH_3 . C(OH)=C . COOH \\ CH_3 . C(OH)=C . COOH \end{array} \rightarrow$$

I.

or one of the oxidation products shown above, viz. α-hydroxyacetoacetic acid, $\alpha\beta$-dihydroxycrotonic acid or its peroxide form, unites with a further molecule of acetoacetic ester, *e.g.* the peroxide form and acetoacetic ester might condense, and the product chiefly losing carbon dioxide yields acetonylacetoacetic ester and to a small extent (0·2 g. from 13 g. acetoacetic ester oxidised) by loss of water, ethyl isocarbopyrotritartrate (II),

$$\begin{array}{l} CH_3 . \underset{\llcorner\!\!-O-\!\!\lrcorner}{C(OH)}=CH . COOH \\ + CH_3 . C(OH)=CH . COOC_2H_5 \end{array} \rightarrow \begin{array}{l} CH_3 . C(OH)=C . COOH \\ CH_3 . C(OH)=C . COOC_2H_5 \end{array} \rightarrow \begin{array}{l} CH_3 . C(OH)=CH \\ CH_3 . C(OH)=C . COOH \\ CH_3 . C(OH)=C . COO \\ CH_3 . C=C . COOC_2H_5 \end{array}$$

II.

The very small amounts of acetic, glyoxylic and oxalic acids detected in the oxidation mixture probably arise from a subsidiary reaction involving a splitting of the enol form of acetoacetic acid at the double bond,

$$\begin{array}{l} CH_3 . C(OH)=CH . COOH \\ \quad\ O \quad\ O \end{array} \rightarrow CH_3 . COOH + CHO . COOH \rightarrow COOH . COOH,$$

the glyoxylic acid being in part further oxidised to oxalic acid.

It appears therefore that the chief oxidation products of acetoacetic acid are α-hydroxyacetoacetic acid, and either acetonylacetoacetic acid or diacetylsuccinic acid. In experiments *in vitro*, the first substance very gradually loses carbon dioxide passing to acetol. If the oxidation of acetoacetic acid *in vivo* produces α-hydroxyacetoacetic acid, it is unlikely that the decomposition into acetol occurs, since this substance although relatively non-toxic, even in moderate doses in phlorrhizinised dogs, causes haematuria and haemoglobinuria [Greer, Witzemann and Woodyatt, 1914]. It seems more probable that the reducing acid would be further oxidised to diketobutyric acid.

Two lines of further change of the diketobutyric acid suggest themselves; (1) hydrolysis with the formation of acetic and glyoxylic acids, or (2) loss of CO_2 with the formation of methylglyoxal:

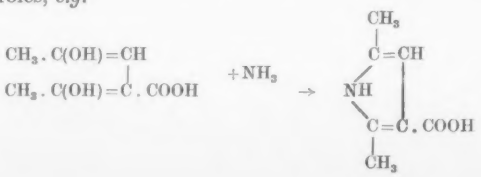
$$(1)\ CH_3 . CO . CO . COOH + H_2O \rightarrow CH_3 . COOH + CHO . COOH$$
$$(2)\ CH_3 . CO . CO . COOH \rightarrow CH_3 . CO . CHO + CO_2.$$

The first of these reactions is in all probability that which occurs when higher members of the series of normal fatty acids undergo β-oxidation. It would result in the loss of two carbon atoms at a time and the process would be in conformity with the β-oxidation hypothesis of Knoop. The second reaction is however not without interest. It also would lead to a shortening of the fatty acid chain by two carbon atoms because the glyoxal derivatives produced by the reaction under the influence of glyoxalase could be converted into α-hydroxy-acids, which in turn by oxidation would lose one carbon atom and yield a fatty acid with an even number of carbon atoms.

In the case of diketobutyric acid itself the second reaction has added significance because it would give rise to methylglyoxal and by the intervention of glyoxalase, lactic acid. This in turn is capable of conversion into glucose. A mechanism is thus provided by which one product of the oxidation of fats may be converted into carbohydrates.

Recently, Knoop and Jost [1924] have shown that following the administration of β-hydroxybutyric acid to dogs, not inconsiderable amounts of lactic acid are found in the urine. They are not inclined to suggest that the lactic acid has arisen through oxidation of the acid administered, partly because it would involve an oxidation in the α-position. The suggestion outlined above would however explain how lactic acid might arise from the oxidation of either butyric, β-hydroxybutyric or acetoacetic acid without any sacrifice of the β-position as the site of the primary change.

One other suggestion of biological interest may be made as a result of this work. Diacetylsuccinic acid and acetonylacetoacetic acid, which are products of the oxidation of acetoacetic acid, react readily with ammonia to form substituted pyrroles, *e.g.*



This reaction is possibly of importance in the production of the pyrrole nucleus of blood pigments and chlorophyll.

Experimental.

Oxidation of acetoacetic ester.

Acetoacetic ester (13 g.) was placed in a small conical flask in a freezing mixture. Sodium hydroxide (40 cc. 10 %) was added and the whole stirred and allowed to stand, when the sodium compound separated. When the temperature reached 0° a few drops of peroxide (30 %) were added and the liquid stirred. The temperature rose immediately and the number of drops of peroxide added was regulated so that the temperature did not rise above 10°. This was repeated until about 9 cc. of peroxide had been added. If a few drops of the solution were withdrawn at any time immediately after the addition of a little peroxide, it was found that Fehling's solution was readily reduced in the cold but that the chromate test for peroxide was negative. After about two-thirds of a molecule of peroxide (per molecule of ester) had been added, the vigorous reaction ceased. If the solution was allowed to stand it became deep red and the first few processes about to be described had to be carried out as rapidly as possible. The solution, after all the peroxide had been added, was saturated with carbon dioxide and extracted three times with ether. The ether was removed from the ethereal extracts by means of a current of air and an oil resulted which, after drying over sodium sulphate and distilling *in vacuo*, was shown to consist of unchanged acetoacetic ester with smaller amounts of ethyl acetate and a yellow oil, B.P. 110°/15 mm. (not identified), together with traces of the reducing substance and acetonylacetone. The aqueous solution remaining after ether extraction was then acidified with dilute hydrochloric acid. As the acid was added an oily substance appeared to separate and this on standing formed a bulky but light crystalline precipitate. This was filtered off. The substance (0·2 g. from 13 g. ester) was insoluble in cold water but soluble in alcohol and in sodium hydroxide, with ferric chloride gave a blue colour, had the equivalent 215, melted at 110°, and corresponded therefore with ethyl isocarbopyrotritartrate. The acid solution after filtration was then extracted seven times with ether, and the ether removed from the combined dried ether extracts by means of a current of air. A considerable amount of yellow oil resulted. If this oil, which is strongly reducing, is allowed to stand in a test-tube at the ordinary temperature, a very gradual development of carbon dioxide takes place over a period of about 20 hours, due to the gradual decomposition of the reducing acid, α-hydroxyacetoacetic acid, into acetol and carbon dioxide. When the oil was converted into semicarbazones by addition of a little water, sodium acetate and semicarbazide hydrochloride, it was noticed that a thick precipitate formed which partially decomposed, giving carbon dioxide and a product relatively very soluble in water. The precipitate was warmed with

water and the more insoluble portion filtered off, and divided by recrystallisation from boiling water into a more soluble portion and a portion only slightly soluble in boiling water. The former semicarbazone after purification by recrystallisation from boiling water and from dilute alcohol melted at 208° and was shown to be acetonylacetoacetic acid semicarbazone, whilst the latter relatively more insoluble substance after recrystallisation from boiling water melted at 230° and corresponded with acetonylacetoacetic ester semicarbazone, being identical with the ethyl 2 : 5-dimethyl-$\triangle^{2:5}$-dihydropyridazine-1-carbonamide-3-carboxylate of Borsche and Spannagel [1904].

Analysis:

Acetonylacetoacetic acid semicarbazone: found; N = 21·2 %; calculated for $C_8H_{11}O_3N_3$; N = 21·2 %.

Acetonylacetoacetic ester semicarbazone: found; N = 18·8 %; calculated for $C_{10}H_{15}O_3N_3$; N = 18·7 %.

Acetonylacetoacetic ester semicarbazone is also much more soluble in acetone than the corresponding compound of the acid and can therefore be extracted from the mixtures by its means.

If, on the other hand, a little of the yellow oil was dropped into cooled ammonia (conc.) and the product allowed to stand, a crystalline precipitate was formed which after filtering off was dissolved in alcohol and precipitated by the addition of water. This substance was ethyl 2·5-dimethylpyrrole-4 carboxylate, M.P. 117°, and corresponded with that of Knorr [1885] obtained by the action of ammonia on diacetylsuccinic ester followed by gentle heating to drive off one molecule of carbon dioxide.

Analysis: found; N = 8·33 %; calculated for $C_9H_{13}O_2N$; N = 8·38 %.

When a further quantity of the oil was rapidly converted in the presence of only a little water into the semicarbazones and the solids filtered off and then treated with water, a decomposition of part of the semicarbazone rapidly took place giving a new semicarbazone which went into solution. Such a solution on standing for about 24 hours deposited crystals which after recrystallisation were shown to be identical by M.P. 187°, mixed melting point with synthetic material, and by analysis, with acetone semicarbazone.

Analysis: found; N = 36·4 %; calculated for $C_4H_9ON_3$; N = 36·5 %.

Since free acetone had been removed from the acids by the method of preparation, this must have arisen by decomposition of an insoluble semicarbazone of acetoacetic acid. This acid was therefore prepared by allowing the ester to stand in contact with the theoretical amount of sodium hydroxide overnight, acidifying and extracting with ether. The acid on treating with sodium acetate and semicarbazide hydrochloride gave a semicarbazone rapidly decomposing and passing into solution from which, on standing overnight, acetone semicarbazone crystallised out. Acetoacetic acid gives also a smaller amount of another product with a characteristic odour which was not identified.

Finally, when some of the oil was boiled for a long time under reflux with

water, or for a short time with potassium carbonate, or when the oil was distilled, a substance was obtained which gave a very slightly soluble semicarbazone, crystallisable from boiling water, which was shown to be identical with acetonylacetone disemicarbazone by M.P. 224° [cf. Posner, 1901], mixed melting point with the synthetic product and by analysis.

Analysis: found; N = 36·6 %; calculated for $C_8H_{16}O_2N_6$: N = 36·8 %.

The disemicarbazone was obtained synthetically for comparison in the following way: diacetylsuccinic ester was prepared by the action of iodine on ethyl sodioacetoacetate in ether solution by the method of Rugheimer [1874] and converted into acetonylacetone by boiling with 20 % aqueous potassium carbonate for an hour [cf. Knorr, 1900]. This was then treated with sodium acetate and semicarbazide hydrochloride, the semicarbazone filtered off and recrystallised from boiling water. The diphenylhydrazone M.P. 120°, and the dinitrophenylhydrazone M.P. 212°, were also prepared and used for comparison.

Analysis:

Diphenylhydrazone: found; N = 19·2 %; calculated for $C_{18}H_{22}N_4$; N = 19·0 %.

Dinitrophenylhydrazone: found; N = 22·0 %; calculated for $C_{18}H_{20}O_4N_6$; N = 21·9 %.

Whenever the yellow oil was allowed to stand for some time, crystals of oxalic acid separated, but complete separation by means of lead and calcium salt methods showed that the amount was small.

Distillation of the oil *in vacuo* always gave a first portion containing acetic acid and having the characteristic strawberry smell of acetol, strongly reducing and readily giving methylglyoxal osazone M.P. 145°.

From the above experiments it appears that ether extraction of the acidified liquid extracts considerable amounts of (1) a very unstable reducing acid, probably α-hydroxyacetoacetic acid, which readily gives acetol, (2) acetonylacetoacetic acid and ester, and (3) acetoacetic acid together with small amounts of oxalic and acetic acids.

The aqueous residue after ether extraction still contains considerable amounts of the reducing acid. It was therefore neutralised to litmus and evaporated *in vacuo* from a water-bath at 50°, the distillate being removed from time to time. The first two-thirds of the distillate contained traces of both acetol and acetonylacetone, but the major part of the reducing body present as the sodium salt was concentrated in the distilling flask and a drop removed therefrom had exceedingly powerful reducing properties in the cold. The receiver was therefore finally changed and the temperature of the bath raised to about 80° and the whole distilled to dryness *in vacuo*. The residue in the distillation flask smelled strongly of acetol. The aqueous distillate reduced Fehling's solution powerfully and was treated with sodium acetate and semicarbazide hydrochloride. An almost immediate precipitation occurred of acetonylacetone disemicarbazone and, after filtering this off, the

solution was allowed to stand for 2 days. A considerable amount of practically pure acetol semicarbazone crystallised out, M.P. 197°.

Analysis: found; N = 32·2 %; calculated for $C_4H_9O_2N_3$; N = 32·06 %.

Acetol was therefore prepared by boiling bromoacetone with anhydrous potassium formate in methyl alcoholic solution under reflux [Nef, 1904] and gave with sodium acetate and semicarbazide hydrochloride a semicarbazone, M.P. 197°, identical with that obtained from the oxidation product and showing no depression of melting point when mixed with it. The presence of acetol in the distillate was confirmed by condensation with *o*-aminobenzaldehyde, when a blue fluorescing substance was obtained [cf. Baudisch and Deuel, 1922] and by preparing therefrom the osazone (M.P. 145°) and the dinitrophenylhydrazone (M.P. 302°, gives a blue colour with alcoholic sodium hydroxide which gradually becomes purple, violet, dull brown [cf. Dakin and Dudley, 1913]).

If the neutralised concentrated solution, instead of being distilled to dryness, was acidified and extracted with ether, a mixture of reducing acid and acetonylacetone and a little oxalic and acetic acids was obtained and the residual solution on continuous ether extraction for 24 hours gave a further quantity of reducing acid and acetic and oxalic acids.

Methylglyoxal was prepared from a sample of ethyl $\gamma\gamma$-diethoxy-α-methylacetoacetate (kindly supplied by Dr Dudley) by the method of Dakin and Dudley [1914], and from dihydroxyacetone both by treatment with dilute sulphuric acid [Pinkus, 1898] and by distilling *in vacuo* with phosphorus pentoxide [Fischer and Taube, 1924]. Methylglyoxal gives a disemicarbazone, M.P. 257°, both when treated with an aqueous solution of semicarbazide hydrochloride and when this solution is buffered either by bicarbonate or by sodium acetate.

Analysis: found; N = 45·3 %; calculated for $C_5H_{10}O_2N_6$; N = 45·2 %.

Ethylglyoxal on the other hand appeared to give a monosemicarbazone, M.P. 235°, on treatment with an aqueous solution of semicarbazide hydrochloride [Dakin and Dudley, 1914].

A point of great interest was the ease with which dihydroxyacetone passed to methylglyoxal. It was found that an aqueous solution of dihydroxyacetone, when mixed with a similar solution of semicarbazide hydrochloride with or without addition of sodium acetate, slowly deposited on standing methylglyoxal disemicarbazone. The passage of the primary alcohol group into a methyl group is interesting but the mechanism does not seem clear. It seems likely that some such reaction may be involved in the production of lactic acid from glucose.

An attempt was made very roughly to follow the oxidation processes quantitatively. Using 26 g. of ester (which corresponds to 20·4 g. acetoacetic acid) and 18 cc. hydrogen peroxide (100 vols.), the following were the weights of the extracts obtained:

(1) after passing carbon dioxide, 3 ether extractions	4·9 g.
(2) after acidifying with hydrochloric acid, precipitated ...	0·4
(3) „ „ „ 7 ether extractions	9·5
(4) after evaporation to ¼ bulk of the neutralised solution, acidification and 7 ether extractions	2·3
(5) after ether extraction for 24 hours	3·1
	20·2 g.

Extract (1) contained chiefly acetoacetic ester together with ethyl acetate, traces of acetol and a yellow oil.

(2) contained ethyl isocarbopyrotritartrate.

„ (3) contained chiefly reducing acid, acetonylacetoacetic acid and ester together with acetoacetic acid, acetol and acetonylacetone, acetic, glyoxylic and oxalic acids.

„ (4) contained same as (3) but the amounts of acetonylacetone and acetic acid were relatively larger.

„ (5) contained reducing acid, oxalic acid, acetonylacetone.

During the processes of evaporation, carbon dioxide, ethyl alcohol, traces of acetol and acetonylacetone were lost.

The main fraction (3) was shaken with 150 cc. water and on conversion into the semicarbazones yielded 6·0 g. dry product. This consisted almost entirely of the semicarbazones of acetonylacetone, acetonylacetoacetic acid and ester, the semicarbazones of acetoacetic acid (as acetone semicarbazone after decomposition) and of acetol remaining in solution. This weight of semicarbazone represents the presence of about 4·8 g. acetonylacetoacetic acid. Acetonylacetoacetic acid, acetonylacetoacetic ester and acetonylacetone are present in the mixture in decreasing amounts. Diacetylsuccinic ester was prepared for comparison and gave a semicarbazone M.P. 203° [Bülow, Riess and Sautermeister, 1905].

$\alpha\beta$-Dihydroxybutyric acid was synthesised by oxidation in dilute solution of crotonic acid by means of barium permanganate [Fittig and Kochs, 1892]. 2·9 g. of this acid was dissolved in 50 cc. water and boiled with 0·14 g. reduced iron and the solution filtered. 14 cc. of 5 % hydrogen peroxide were added to the solution in an ice-bath. No rise of temperature occurred and the solution was therefore allowed to stand at the ordinary temperature. The temperature after about half an hour had risen to 20°. The liquid was cooled to 12° and allowed to stand about 4 hours. Samples removed from the liquid a quarter of an hour after adding the peroxide, on making alkaline, gave no reduction with Fehling's solution in the cold but after standing for a longer time the liquid readily caused reduction in the cold. The aqueous solution was powerfully reducing after standing 24 hours, and on treatment with *p*-nitrophenylhydrazine in acetic acid solution immediately gave a dinitrophenylhydrazone which on precipitation from hot nitrobenzene solution with toluene melted at 304°, gave a blue colour with alcoholic sodium hydroxide and corresponded with methylglyoxal dinitrophenylhydrazone.

SUMMARY.

(1) The oxidation of the sodium compound of acetoacetic ester or acid in the cold by means of hydrogen peroxide yields, as primary product, α-hydroxyacetoacetic acid (or $\alpha\beta$-dihydroxycrotonic acid). This readily loses carbon dioxide, forming acetol.

(2) In addition, by subsidiary reactions, the following substances are produced; diacetylsuccinic acid, acetonylacetoacetic acid, acetonylacetone, acetic acid, glyoxylic acid and oxalic acid. Of these, acetonylacetoacetic acid is formed in considerable amount.

(3) The mechanism of the reaction is discussed in relation to the later stages of the β-oxidation process, the possible derivation of carbohydrate from acetoacetic acid and a mode of origin of substituted pyrroles in living organisms.

We desire to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this investigation.

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XI. THE TYROSINASE-TYROSINE REACTION.

IV. NOTE ON THE IDENTITY OF TYROSINASE FROM DIFFERENT SOURCES.

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(*Received December 31st, 1925.*)

In 1923 Raper and Wormall described three stages which may be sharply distinguished in the reaction between potato tyrosinase and tyrosine [1923]. The first of the stages is the production of a red substance; in the second stage the red substance changes spontaneously into a colourless substance, this being best observed in an inert gas or *in vacuo*; finally, on exposure to oxygen the colourless substance blackens to form melanin, this being the third stage. It was shown that the enzyme is necessary only for the first stage of the reaction, *i.e.* the oxidation of the tyrosine to the red substance. If at this stage the enzyme be removed with "colloidal iron" the red substance still changes to the colourless one and the subsequent oxidation of this to melanin also takes place. These observations were made solely with tyrosinase derived from potatoes. Gortner [1910, 1924], using an animal tyrosinase prepared from the mealworm (*Tenebrio molitor*), was unable to observe the change from the red substance into the colourless one, even after many days. This made it appear that the enzyme from mealworms was different from that present in potatoes, in that the red substance produced in the two cases had different properties. We have therefore repeated Gortner's experiment adhering exactly to his directions for the preparation of the enzyme and the carrying out of the experiment, but we have failed completely to note any difference in the behaviour of the red substance whether it is produced by the potato or mealworm enzyme. Thinking that the p_H of the solution might influence the reaction, the effect of this on the rate of change of the red substance into the colourless one has been investigated. At p_H 8 the change is rapid and it becomes considerably slower at p_H 6 but even at this p_H the change is complete at the ordinary temperature in 24 hours. This therefore could not explain the difference between Gortner's experiment and our own, so that we must conclude that some condition, not described by Gortner, which is essential for stabilising the red substance, has been overlooked in his description. As a further proof of the identity of the two

enzymes, the limits of p_H at which the mealworm enzyme acts have been determined and they are substantially the same as for the potato enzyme. In addition no difference has been detected between the soluble and insoluble tyrosinase of the mealworm, although Gortner [1910] states that the latter has no action on quinol. In order to determine whether tyrosinase prepared from a fungus displayed the same properties as the enzyme from the potato and mealworm, *Agaricus dryophilus* has been used as a source of the enzyme. The red substance produced from tyrosine by this preparation displayed exactly the same properties as that obtained by the action of the other two enzymes. The p_H on the acid side of the neutral point at which the action of the *Agaricus* enzyme was inhibited was, however, lower than with either the potato or mealworm enzyme. Since the last two preparations contain protein which is precipitated along with the tyrosinase at p_H 5, whereas no precipitate forms in the *Agaricus* preparation at this p_H, we attribute the difference in the limits of reaction at which the enzymes display activity to this fact. When tested on a series of phenols in buffered solutions at p_H 7, no differences were found between the action of the mealworm and potato enzymes. Incidentally, the rapid change of the red substance into the colourless one at p_H 8 explains why melanin formation takes place most rapidly at this degree of reaction when tyrosinase acts on tyrosine. At p_H 8 the colourless substance, rapidly produced from the initial red pigment, is just as rapidly oxidised at this p_H into melanin, whereas at p_H 6 both processes are much slower.

Experimental.

The insoluble enzyme was prepared from 35 g. *Tenebrio molitor* according to the directions of Gortner [1910], and emulsified in 200 cc. chloroform water.

Exp. 1. To 50 cc. of a saturated solution of tyrosine in a phosphate buffer mixture at p_H 6, 10 cc. of the enzyme suspension were added. The solution was shaken at intervals and had become deep red in 5 hours. It was filtered, a few drops of 10 % acetic acid added and boiled. The filtrate became colourless on boiling and blackened almost immediately on making slightly alkaline with sodium carbonate. This behaviour is identical with that described by Raper and Wormall [1923] using potato tyrosinase.

Exp. 2. p_H *range.* Portions of 25 cc. each of saturated, buffered tyrosine solution, ranging from p_H 4 to 9·5, were set up in each of a series of flasks in two sets. To the flasks of one set, 2 cc. of the mealworm enzyme were added and to the other, 2 cc. of freshly dialysed potato juice. Examined after 24 hours there was no qualitative difference between the two sets. The maximum coloration was produced at p_H 7·8 and at p_H 4 and 9·5 there was no action.

The "soluble" tyrosinase prepared from mealworm by Gortner [1910] was also examined as to its p_H range. The preparation obtained by us had

only a feeble action on tyrosine but this was most clearly marked around p_H 8. It should be noted that the filtrate from the "insoluble" enzyme prepared according to Gortner is at first turbid. It was our practice to return this to the funnel until a clear solution came through and not to precipitate straight away with ammonium sulphate. If the first runnings are included in the "soluble" enzyme solution it is obviously contaminated with the "insoluble" enzyme. This difference in procedure no doubt explains our lack of success in obtaining very active preparations of the "soluble" enzyme. As a matter of fact, the "insoluble" enzyme is quite soluble if it be suspended in water rendered faintly alkaline with ammonia and it only owes its insolubility when prepared according to Gortner's method to the fact that on standing it becomes slightly acid. We suggest therefore that there is no real difference between Gortner's "soluble" and "insoluble" tyrosinase from the mealworm.

Exp. 3. A 1 % solution of quinol buffered at p_H 7·4 was divided into three portions. To these 3 cc. of potato tyrosinase, mealworm tyrosinase (soluble) and mealworm tyrosinase (insoluble) were added respectively. After 24 hours they were all brownish red in colour. Gortner describes the insoluble enzyme as having no action on quinol in 72 hours, but since the p_H of the solution was not adjusted this is a sufficient explanation of the discrepancy between our result and his. A similar experiment to that just described was carried out with the "insoluble" enzyme and the potato enzyme, using phenol, *p*-cresol, resorcinol and catechol as substrates, but no qualitative difference could be detected between the action of the two preparations. Both were without action on resorcinol.

Exp. 4. *Influence of p_H on decolorisation of the red compound.* Two 50 cc. portions, A and B, of a saturated tyrosine solution, buffered at p_H 6, were taken. To A, 3 cc. of freshly dialysed potato juice were added and to B, 3 cc. of the "insoluble" *Tenebrio* suspension. When a marked red colour had developed in both flasks, the contents were filtered through fine paper and paper pulp until the filtrates were clear. A little "dialysed iron" was added to the A filtrate and it was again filtered. Three samples of 5 cc. each from the two filtrates were now placed in Thunberg tubes and the p_H adjusted as follows:

(*a*) 5 cc. filtrate + 1·03 cc. water; p_H 6.

(*b*) 5 cc. filtrate + 0·43 cc. water + 0·6 cc. *N*/5 NaOH; p_H 7.

(*c*) 5 cc. filtrate + 1·03 cc. *N*/5 NaOH; p_H 8.

The tubes were immediately evacuated by a water-pump and then placed in a water-bath at 37° in which the colour changes were followed. In 4 minutes both the tubes at p_H 8 were colourless. In 13 minutes the tubes at p_H 7 were colourless whilst the tubes at p_H 6 were still pale pink. In the last two the colour faded much more slowly than in the tubes at p_H 7 and 8. After 20 minutes the tubes were taken out of the bath at 37° and left at the ordinary temperature. In 18 hours the tubes at p_H 6 were also colourless. Air was

now admitted to all the tubes and those at p_H 6 and 7 were adjusted to 8. At the end of 48 hours all the tubes showed an equally black pigmentation.

Exp. 5. 3 cc. of the insoluble enzyme preparation were added to 50 cc. of saturated tyrosine solution at p_H 6. A well-marked red colour had developed in 4½ hours. The solution was filtered through fine paper [cf. Gortner, 1924] and gave a clear red filtrate. This was placed in the dark at room temperature for 24 hours when all traces of red colour had disappeared, the solution being now blackish brown. This experiment has been repeated several times always with the same result. In the similar experiment described by Gortner the red colour persisted for 18 days. The difference is not due to the use of a phosphate buffer since the same result is obtained when no buffer is used. It is apparently due to some unknown factor which we have been unable to discover.

Exp. 6. Several specimens of *Agaricus dryophilus* were ground up with chloroform water and allowed to macerate for 3 days. The solution contained an active tyrosinase. A portion was filtered and an experiment similar to number 4 was carried out using this as the tyrosinase preparation. The results were identical with those obtained with the potato and *Tenebrio* enzymes. This enzyme preparation when tested as to its p_H range, unlike the *Tenebrio* and potato enzymes, showed some action at p_H 4, but the maximum was around p_H 8 as with the other tyrosinases. It was not precipitated at p_H 4 or 5.

Summary.

(1) Preparations of tyrosinase from the potato, the mealworm (*Tenebrio molitor*) and *Agaricus dryophilus* produce a red compound from tyrosine which has the same properties in each case. There is thus no reason to believe that different tyrosinases have different actions on tyrosine.

(2) No differences could be detected between the actions of the mealworm and potato enzymes on quinol, phenol, *p*-cresol, resorcinol or catechol nor in the p_H range at which they act.

We wish to express our thanks to the Government Grant Committee of the Royal Society for a grant in aid of this work.

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XII. THE PIGMENTS OF BUTTERFLIES' WINGS.

I. *MELANARGIA GALATEA.*

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(*Received December 15th, 1925.*)

The wings of the "Marbled White," *Melanargia galatea*, a Satyrine butterfly not uncommon in southern England, bear a pattern of black markings on a ground varying from creamy white to pale yellow. It was observed by Coste [1890] and various others that this ground colour was reversibly changed to a bright yellow by the action of alkalies or ammonia vapour, the colour returning to the normal pale tint when the alkali was washed away or the vapour allowed to diffuse into the air. This suggested to the writer that the pigment might be a flavone or flavonol, and when Sir F. G. Hopkins very kindly presented some 200 specimens of this butterfly from his own collection, an attempt was made to isolate the pigment in the hope of proving this.

The wings, carefully removed from the bodies, were first extracted with ether in a Soxhlet, and the yellow filtrate shaken with dilute aqueous ammonia, into which most of the colour passed. A certain amount of pigment insoluble in ammonia was, however, also present, and was divided between light petroleum and methyl alcohol. The fraction soluble in light petroleum gave the colour reactions with H_2SO_4 and HNO_3 characteristic of lipochromes, and showed the absorption spectrum of carotene. The much smaller amount of pigment dissolving in the methyl alcohol gave no reactions or spectrum to support the assumption that it was xanthophyll. Lipochromes have repeatedly been found in the lymph of larval Lepidoptera, for example by Poulton [1885], and in the wings of the imagines by Wieland and Schöpf [1925] in *Gonepteryx rhamni*. They occur probably in the spaces between the two laminae of the wing, rather than in the chitinous scales.

The wings were then extracted with alcohol, which removed most of the pigment, and the filtrate diluted with water and freed from alcohol by distillation under reduced pressure. They were further extracted with aqueous ammonia, which was filtered and neutralised and then repeatedly shaken up with ether to extract the pigment. The aqueous layer, however, retained a yellow colour, which became deeper in alkaline solution and was probably

mainly due to the presence of decomposition products of a tarry nature. Alkaline solutions of the pigment rapidly turn dark in air, and on neutralising yield a brown amorphous substance, possibly analogous to quercetinamide.

The various lots of pigment were washed several times by extracting with ether from neutral aqueous solutions and removing from the ether with ammoniacal solutions. They were then united in a concentrated alkaline solution, and the pigment precipitated by acidification. The precipitated pigment was dissolved in alcohol and recrystallised from this solvent three times, precipitation being aided by the addition of distilled water to the filtered solution.

In this way about half a milligram of a solid product was obtained, in the form of a yellow crystalline powder, somewhat sandy in appearance. It was readily soluble in acetic acid or hot alcohol, sparingly soluble in ether, and very slightly soluble in water. It dissolved readily in alkalies or in concentrated mineral acids with a bright yellow colour. The alcoholic solution gave a deep yellow precipitate with lead acetate, and with ferric chloride a greenish brown solution, fluorescing green but changing to a uniform deep red on warming. When boiled with magnesium powder and a few drops of HCl it yielded a pink filtrate which became bright yellowish green on making alkaline (formation of an allocyanidin). The melting point obtained was 253° (corrected), liquefaction being accompanied by partial decomposition. The molecular weight was determined by Rast's camphor method, 0·039 mg. pigment in 0·988 mg. camphor of melting point 174°, giving a depression of the melting point of 4·8°: this indicates a molecular weight of 313 ± 30, the probable error with such small quantities and so slight a depression being not less than 10 %. The writer does not hesitate to say that these properties definitely lead to the conclusion that the pigment is a flavone or flavonol. Whilst no claim of identity can be made, the described properties of quercetin (molecular weight 302, melting point just over 250° with partial decomposition, and colour reactions similar to those described above, except that the lead salt is spoken of as orange or brick-red) agree fairly well with those of the substance discussed.

It is probable that the flavone is obtained by the larva from its food plants (*Dactylis glomerata*, *Phleum pratense* and other grasses), but the writer has been unable to find any reference to the flavones of these plants in the literature. Nor is anything known of the behaviour of such substances in the animal body, except that euxanthone passes partly unchanged through the mammalian body to reappear in the urine ("Indian yellow"). Palmer and Knight [1924] obtained from a Hemipteron, *Leptocoris trivittatus*, aqueous solutions of a yellow colour which was intensified by alkalies, and which they considered to be due to a flavone-like pigment; but they did not show that it was derived from plants. The writer hopes to continue the research in this direction.

SUMMARY.

A yellow pigment having the properties of a flavone or flavonol has been isolated from the wings of the Marbled White Butterfly, *Melanargia galatea*.

My best thanks are due to Prof. Sir F. G. Hopkins for his continued interest in this research, and especially for providing me with the butterflies used.

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XIII. STUDIES IN NITROGEN AND SULPHUR METABOLISM.

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(*Received December 16th, 1925.*)

THE experiments to be described were undertaken to gain further insight into the course of protein metabolism and to extend the work recorded in a previous paper [Wilson, 1925] by the investigation of the following points:

1. What capacity has gelatin for sparing body protein when ingested during starvation?

2. Is the gelatin under such circumstances used immediately for energy purposes or does it become incorporated in the cell and influence protein metabolism?

3. What is the influence of meat extractives on protein metabolism when the body is on a nitrogen-free diet?

4. What is the influence of excess water ingestion on protein metabolism?

The quantitative effect of gelatin on the nitrogen output in starvation has been repeatedly investigated [Voit, 1881; Kirchmann, 1900; Krummacher, 1901]. These investigators found that gelatin, no matter the amount given, never reduced the fasting nitrogen output more than 37 %. The sparing effect of gelatin however on the endogenous nitrogen output, *i.e.* when the body is on a carbohydrate-fat diet, is almost nil [Robison, 1922]. The present experiments were directed chiefly to the qualitative effect of gelatin on the nitrogen metabolism in starvation.

The experiments were carried out on myself as subject and the 24 hours' urine was collected and analysed for total nitrogen, total sulphur, ammonia urea, uric acid and phosphorus as described in the previous paper.

In Exp. 1 a nitrogen-free diet of tapioca and olive oil was taken until the nitrogen output in the urine amounted to about 3 g. *per diem* and represented approximately the endogenous wear and tear quota. Four days of starvation followed, on the second day of which 70 g. gelatin containing 11·8 g. nitrogen and 0·3368 g. sulphur with a S : N ratio of 1 : 35 were ingested in three portions during the course of the day. At the end of starvation the nitrogen-free diet was resumed until the nitrogen output had returned to its original level. Exp. 2 was a control in which no gelatin was taken. The excess nitrogen output above the wear and tear quota was found

Table I.

Date 1924	Diet	Total N g.	Urea-N g.	Ammonia-N g.	Uric acid mg.	Total S g.	Total P_2O_5 g.	S : N
Exp. 1.								
Feb. 4	Basal	4·900	3·416	0·252	327	0·341	1·564	1 : 14·3
,, 5	,,	3·808	2·425	0·235	295	0·253	1·150	1 : 15·5
,, 6	,,	3·528	2·184	0·252	262	0·231	0·923	1 : 15·2
,, 7	Starvation	3·248	2·072	0·168	270	0·247	0·979	1 : 13·1
,, 8	70 g. gelatin	7·364	5·510	0·369	337	0·407	0·781	1 : 18·1
,, 9	Starvation	8·176	6·597	0·319	252	0·390	1·547	1 : 20·9
,, 10	,,	9·324	7·974	0·482	257	0·555	2·059	1 : 16·8
,, 11	Basal	6·636	4·592	0·392	440	0·357	0·682	1 : 18·3
,, 12	,,	3·920	2·240	0·664	350	0·253	0·866	1 : 15·5
,, 13	,,	3·360	2·072	0·298	307	0·231	0·823	1 : 14·5
Exp. 2. 1925								
Feb. 23	Basal	5·096	3·584	0·337	389	0·357	1·405	1 : 14·2
,, 24	,,	4·458	3·178	0·350	412	0·324	1·391	1 : 13·7
,, 25	,,	4·144	2·968	0·336	389	0·346	1·278	1 : 11·9
,, 26	,,	4·816	3·364	0·336	350	0·527	0·951	1 : 9·1
,, 27	,,	11·008	9·394	0·742	389	0·774	2·442	1 : 14·3
,, 28	,,	13·196	11·920	1·260	475	0·802	3·436	1 : 16·4
Mar. 1	,,	11·872	9·512	1·426	587	0·819	3·138	1 : 14·4
,, 2	,,	8·120	6·440	0·784	637	0·484	1·058	1 : 16·7
,, 3	,,	4·50)	3·010	0·546	487	0·423	0·951	1 : 10·6
,, 4	,,	3·752	2·548	0·420	362	0·357	1·192	1 : 10·5

by subtracting the nitrogen output on the day before starvation from that of the starvation and subsequent days until the urinary output had reached the original value. Calculating we find:

Excess nitrogen above basal value = 17·5 g.
,, sulphur ,, ,, = 0·8244 g.
S : N ratio of excess = 1 : 21·2
Nitrogen ingested = 11·8 g.
Sulphur ,, = 0·3367 g.

The real loss of nitrogen above the basal value is therefore 5·7 g. and of sulphur 0·4877 g. with a S : N ratio of 1 : 11·7.

The total nitrogen output on the four starvation and two post-days (not subtracting the basal value) is 38·667 g. and that of sulphur 2·209 g., the S : N ratio being 1 : 17·8. If the nitrogen and sulphur of the gelatin are subtracted from these figures the total loss to the body is 28·8 g. nitrogen and 1·877 g. sulphur with a S : N ratio of 1 : 14·3, a figure which corresponds with the S : N ratio of muscle. It is clear that the tissues have not metabolised a sulphur-rich protein in order to improve the quality of the sulphur-poor gelatin. In Exp. 2, the control, we find the balance:

Excess nitrogen above the basal = 26·64 g.
,, sulphur ,, ,, = 1·75 g.
S : N ratio of excess = 1 : 16·2

The total output on the other hand is 53·512 g. nitrogen and 3·8 g. sulphur with a S : N ratio of 1 : 13·9, approximately that of muscle tissue. It would seem that the protein tissue utilised in starvation was muscle and that the giving of some other protein such as gelatin does not cause the body to break

down tissue protein of a different nature. The average S : N ratio of Cathcart's subject, Beauté, for the 14 starvation and 3 post-days is 1 : 13·7 [Cathcart, 1907]. Benedict's subject, Levanzin, for the first 14 days of his fast had a S : N ratio of 1 : 15·7, which Benedict considers to be due possibly to the breakdown of sulphur-poor material [Benedict, 1915].

It seems anomalous that the total nitrogen output in Exp. 2, when no gelatin was taken, should be greater than in Exp. 1. The result might suggest that, contrary to all previous findings, gelatin has a considerable sparing action on tissue breakdown in starvation. A possible and indeed probable explanation is the fact that in Exp. 1 the subject had just recently carried out a series of starvation experiments and was hence in a nitrogen-poor condition. Exp. 2 was carried out a year later following a period of normal diet. In addition it is possible that in starvation the absolute metabolism of protein is reduced. Hawk, Howe and Mattill [1911] noted such a reduction in a dog, the nitrogen output during a second fasting period being much lower than during the first. Benedict [1919] states that after a period of restricted diet the total metabolism is reduced; he considers that the loss of nitrogenous material from the fluids bathing the cells may diminish the specific stimulus to metabolism. In Exp. 2 the body had evidently a store of some labile nitrogen which was wanting in Exp. 1. From the great difference in the total nitrogen loss in the two experiments it is probable that gelatin has exercised some sparing action although it is difficult to say to what extent this has taken place.

An examination of the S : N ratio of the days following the taking of the gelatin shows that it was being metabolised up to the last day of the experiment. The ratio falls only to 1 : 14·5 on the last day of the experiment. In the control it has reached on the third post-day a value of 1 : 10·5. In a similar experiment (Exp. 1) in the previous paper [Wilson, 1925] it reached the value 1 : 10·4. A rise to 1·99 was also observed in Cathcart's subject, Beauté, on the third day of starch and cream following a fast [Cathcart, 1907]. The conclusion to be drawn is that some of the gelatin has been retained in the tissues for 6 days. It seems unlikely that it could have been circulating in the amino-acid form during this period and the conclusion may be warranted that it has been retained in some more complex form and that later this material became metabolised, possibly playing a part where a biologically complete protein was not essential.

As regards the uric acid output it will be seen that it is roughly proportional to the total nitrogen output; that is, it is greater in Exp. 2 than in Exp. 1. Another striking point is the immediate rise in uric acid output on the first post-day when the diet was resumed. This favours the idea that carbohydrate is necessary for the formation of the purine ring as suggested by Cathcart [1922]. The highest uric acid output was observed on the second post-day in Exp. 2 and is possibly accounted for by the fact that acetone was still present in the urine on the first post-day; the carbohydrates ingested

may thus have been stored on this day either as glycogen or possibly as a hexosephosphoric ester (it will be noted that the phosphorus output dropped on the first post-day in Exp. 2 from 3·13 to 1·05 g.). The sharp drop in the phosphorus output is partially accounted for by the reduction in acidosis, as is seen from the fall in the ammonia output.

The conclusions as to the influence of meat extractives on protein metabolism have been rather conflicting. Burgi [1904] found, on giving Liebig's extract to starving dogs, that it exerted no sparing action on the nitrogen output and in addition was mostly excreted unchanged, 5 % only being unaccounted for. Rubner [1904] came to the same conclusion but qualified it by stating that if the tissues are poor in certain constituents present in the extract a retention might occur. He also showed that the carbon-poor extractives were excreted before the carbon-rich. Thompson [1910] stated that extractives improve the digestibility of the food taken with them and promote nitrogen retention. In the present investigations (Exp. 3, Table II) 25 g.

Table II.

Exp. 3.

Date 1924	Diet	Total N g.	Urea-N g.	Ammonia-N g.	Uric acid mg.	Total S g.	Total P_2O_5 g.	S : N
May 25	N-free basal	5·152	3·711	0·241	350	0·522	1·630	1 : 9·8
,, 26	,,	3·948	2·844	0·207	342	0·341	1·164	1 : 11·5
,, 27	,,	3·388	2·290	0·202	330	0·258	0·956	1 : 13·1
,, 28	,,	2·744	1·717	0·274	242	0·247	0·908	1 : 11·2
,, 29	,,	3·416	0·789	0·252	580	0·313	2·101	1 : 10·9
	+25 g. extractives							
,, 30	,,	2·917	1·601	0·190	387	0·231	1·562	1 : 12·2
,, 31	,,	2·576	1·400	0·168	350	0·247	1·107	1 : 10·4

meat extractives were superimposed on a nitrogen-free diet when the daily nitrogen output had fallen to 2·744 g. It will be noted that the output rose on the day of ingestion to 3·416 g. and on the second post-day had fallen below the value of the pre-day. As the basal value on the day before taking the extractives might be too high (the endogenous output having scarcely reached its minimum figure) the basal value was taken as the mean between the pre-day and the last post-day. The excess nitrogen output due to the taking of the extractives was 1·029 g. and the sulphur 0·0494 g. with a S : N ratio of 1 : 20. The extractives taken contained 2·689 g. nitrogen and 0·1236 g. sulphur with a S : N ratio of 1 : 19·7. The amount stored is thus 1·661 g. nitrogen and 0·0742 g. sulphur with a S : N ratio of 1 : 22·0. If on the other hand the pre-day figure alone be utilised as the basal value a retention above the basal value of 1·844 g. nitrogen and 0·742 g. sulphur with a S : N ratio of 1 : 24·8 is found. Under the conditions of the experiment the tissues have retained a material which is definitely sulphur-poor. It will be noted that, on the nitrogen-free diet, the S : N ratio tends to rise, *e.g.* Exps. 2 and 3; this signifies a relative retention of nitrogen or, expressed otherwise, a lag in the excretion of nitrogen behind the sulphur. It seems extremely probable that it is this tendency for nitrogen in preference to sulphur retention which

has favoured the storing of sulphur-poor material from the ingested extractives. It will also be noted that the urea output on the day of ingestion is markedly reduced both relatively and absolutely. The relative decrease is to be explained by the elimination in the urine of nitrogen-containing extractives; the uric acid output, for instance, is doubled. The absolute decrease is peculiar because on the first post-day it returns to its pre-day value; there is no compensatory increase in the ammonia output to account for it. The suggestion is made that the extractives must have influenced the formation of urea, probably by diminishing the deaminising processes. The conclusions to be drawn are, firstly, that under suitable conditions, such as nitrogen starvation of the tissues, meat extractives can be stored up to 70 % of their nitrogen content, and, secondly, that the extractives do not stimulate protein metabolism but, on the contrary, seem rather to inhibit it and thus favour storage.

The effect of water ingestion on protein metabolism has been a subject of investigation since Voit's time. Voit [1881] noted an increase in the urea output of a dog after the ingestion of excess water. He concluded that it was not due to a washing out as there was no increase in the salt output which, owing to its higher concentration in the blood than urea, should have been increased if washing out were the cause. His conclusion was in favour of an increase in protein catabolism. Heilner [1907] came to the same conclusion and noted that the effect was not observed in a well-fed animal but only in a starving one. Hawk [1910] in his experiments on human subjects showed that excess water ingestion improved digestion and assimilation and promoted the retention of protein. Orr [1914] found that it increased the absolute and relative amount of urea and concluded that water stimulated the catabolism of protein and promoted subsequent retention of nitrogen. The present experiments are an attempt to elucidate whether water ingestion causes a mere washing out of the tissues or stimulates protein metabolism. It was thought that the consideration both of the sulphur and the nitrogen output would aid the solution of the problem. Three series of experiments were therefore performed. In series A (Table III) a constant diet of bread and cheese was taken until nitrogen equilibrium was obtained. The S : N ratio was not very constant but had an average of 1 : 14. Two litres of water were ingested on two separate days and the effect on the output noted. On the second water-drinking day the day and night urines were collected and estimated separately. It will be noted that on each occasion the ingestion of water brought about a rise in the nitrogen output and a fall in the S : N ratio. An examination of the day and night urine of the second water-drinking day shows that the increase in nitrogen output occurred during the day. On each occasion the S : N ratio rose on the following day along with a retention of nitrogen. The sulphur on the other hand scarcely shows any rise at all; nevertheless on the subsequent day there was a retention of sulphur. The results of these experiments were not definite but if anything,

Table III.

Series A.

Date 1924		Diet	Urine cc.	Total N g.	Uric acid mg.	Total S g.	Total P_2O_5 g.	S : N
Nov.	3	Basal	660	8·592	488	0·615	2·399	1 : 13·9
„	4	„	640	8·540	512	0·604	2·428	1 : 14·1
„	5	„	640	9·072	540	0·654	2·442	1 : 13·8
„	6	„	630	8·932	546	0·665	2·338	1 : 13·4
„	7*	„	1600	9·982	537	0·670	2·712	1 : 14·7
„	8	„	750	8·708	540	0·604	2·634	1 : 14·3
„	9	„	640	8·848	536	0·610	2·385	1 : 14·5
„	10	„	550	8·736	545	0·621	2·442	1 : 14·0
„	11	„	560	8·764	505	0·626	2·343	1 : 13·9
„	12* Day	„	1196	5·339	231	0·317	1·378	1 : 16·8
	Night		630	4·284	—	0·383	1·278	1 : 13·6
	Total		1826	9·625	—	0·630	2·656	1 : 15·2
„	13	„	900	8·036	461	0·588	2·201	1 : 13·7
„	14	„	700	8·492	526	0·560	2·560	1 : 14·4
„	15	„	700	8·456	532	0·615	2·428	1 : 13·7

Series B.

Date 1925		Diet	Urine cc.	Total N g.	Urea-N g.	Ammonia-N g.	Uric acid mg.	Total S g.	Total P_2O_5 g.	S : N	Urea-N % of total N
Oct.	23	Basal	900	9·800	8·287	0·494	547	0·593	2·428	1 : 16	84
„	24	„	1000	9·772	7·985	0·414	500	0·637	2·130	1 : 15·3	81
„	25	„	800	9·800	7·940	0·571	612	0·665	2·272	1 : 14·7	80
„	26†	„	2600	10·080	8·636	0·604	615	0·676	2·424	1 : 14·9	85
„	27	„	800	9·204	7·834	0·398	370	0·632	2·130	1 : 14·5	85
„	28†	„	3200	10·136	8·758	0·538	520	0·615	2·840	1 : 16·9	86
„	29	„	900	8·988	7·431	0·437	472	0·626	2·456	1 : 14·3	82
„	30	„	800	9·268	7·558	0·364	487	0·593	2·399	1 : 15·7	81

Series C.

Nov.	2	Basal	700	7·868	6·204	0·375	475	0·588	2·499	1 : 13·3	78
„	3	„	700	7·672	6·333	0·302	370	0·604	2·485	1 : 12·6	82
„	4	„	700	7·948	6·661	0·325	421	0·670	2·683	1 : 11·8	80
„	5†	„	2940	8·148	7·022	0·538	486	0·643	2·683	1 : 12·6	86
„	6	„	1100	7·098	5·630	0·370	487	0·610	2·619	1 : 11·6	79
„	7	„	980	7·364	6·338	0·330	482	0·626	2·627	1 : 11·6	86
„	8	„	620	7·600	6·120	0·347	555	0·648	2·556	1 : 11·7	80
„	9†	„	2300	8·064	7·168	0·588	516	0·626	2·641	1 : 12·8	88
„	10	„	700	6·748	5·432	0·319	487	0·626	2·201	1 : 10·7	80
„	11	„	660	7·128	5·678	0·342	482	0·621	2·485	1 : 11·4	79

* 2 litres water drunk. † 2800 cc. water drunk.

except for the failure of the sulphur to increase, they favour the theory that it is a mere washing out process. Further experiments were carried out in which the basal S : N ratios were definitely divergent. Series B (Table III) consisted of a diet of bread and gelatin which had a low S : N ratio; series C consisted of a diet of bread and dried eggs with a high ratio. In addition urea and ammonia were estimated in series B and C. On each diet there were two water-drinking days when 2800 cc. water were taken. It will be noted that with the low S : N in series B the ratio fell on both water-drinking days but most definitely on the second water day. The slight change of

S : N ratio on the first day may be due to the fact that the increased nitrogen output is only 0·208 g. The urea output on the other hand increased 0·6 g. but the sulphur increased only 11 mg. On the second water day the ratio definitely fell. Although the nitrogen output increased about 0·8 g. the urea output increased over 1 g. The sulphur output on the other hand was reduced. In series C with the high S : N ratio the same phenomena are seen, a fall in the ratio and a rise in the nitrogen output, whilst the urea is increased both absolutely and relatively. The sulphur is decreased in both cases and there is a tendency to retention on the subsequent days which more than compensates for the loss on the day of water ingestion. It was hoped, if the effect of water was to stimulate tissue metabolism, that the gelatin series would show a rise; *i.e.* assuming that the water stimulates the breakdown of muscle tissue with a S : N ratio of 1 : 14·3, which would clearly tend to raise the low ratio of the gelatin series. In series C on the other hand one would expect the ratio to fall if tissue breakdown occurs. If, however, water stimulated the metabolism of the protein ingested at the time the ratio should remain the same. If it is a case of washing out, on the other hand, the ratio should fall in each experiment. Although the results would seem to favour the last view, there are three features which require explanation, firstly, the sulphur output nearly always tends to fall; secondly, the increase in the urea-nitrogen is greater than that of the total nitrogen; thirdly, the subsequent storage, which more than compensates the previous loss. The increase in the urea would suggest that the ingestion of water brings about a qualitative change in metabolism. The increased formation of urea must have been from materials which normally would have been excreted in some other form as residual nitrogen, amino-acids, purines etc. Howe, Mattill and Hawk [1912] have shown that the purine output is diminished in a starving dog after water ingestion. Orr [1914] concludes that water ingestion increases the catabolism of protein and promotes nitrogen retention. The present experiments also seem to show that water causes an increased catabolism of the nitrogen moiety of the protein molecule and simultaneously promotes storage. The fact of a small reduction in the sulphur output on the water day would suggest that the storage of sulphur commences on that day and favours the suggestion made in the previous paper [Wilson, 1925] that it is the sulphur which is apparently stored first.

Summary.

1. A protein such as gelatin, when ingested during starvation, can be retained in part for at least six days. It is probable that the retention takes place in some complex form and not as the free amino-acids.

2. Meat extractives ingested during nitrogen starvation are in part retained. The material retained does not correspond in composition to muscle

tissue. Extractives further seem to exercise some kind of inhibitory influence on nitrogen catabolism.

3. Excess water ingestion increases the output of nitrogen in the urine by stimulating the catabolism of the nitrogen moiety of the protein molecule. The capacity of the tissues to retain protein also seems to be stimulated.

4. Further evidence is adduced in favour of the view that in the storage of protein the sulphur is retained first.

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XIV. THE CHEMICAL NATURE OF THE SUBCUTANEOUS FAT IN THE NORMAL AND SCLEREMATOUS INFANT.

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Sclerema neonatorum is a rare but interesting condition peculiar to infants and is characterised by the appearance of hard well-defined thickenings of the subcutaneous tissue. It is with the material composing these swellings that this investigation is concerned. Frozen sections of the tissue show large numbers of sheaves of crystals which, seen through crossed Nicol prisms, are doubly refracting. It was the observation of the presence of these crystals which led to this work being undertaken, for their doubly-refracting nature suggested that they were probably cholesterol or its esters. Subsequent work proved that this view was probably incorrect, and the work finally took the form of an enquiry into the nature of the subcutaneous fat in normal children and in those suffering from sclerema.

Observations of the behaviour of these crystals in the frozen sections of sclerema cases towards various solvents under the polarising microscope were made. By direct observation in this way, they appeared to be insoluble in ether, acetone, light petroleum, pyridine and alcohol; likewise in acid alcohol, alcoholic sodium hydroxide, water, hydrochloric acid and sodium hydroxide. It is to be noted, however, that the temperature of a volatile solvent rapidly evaporating on a slide is probably well below 0°. On slightly warming, the crystals were soluble in ether, acetone, light petroleum and pyridine, but insoluble in water, aqueous hydrochloric acid and sodium hydroxide; in the other reagents they were slightly soluble. Between 40° and 50° the crystals melted, to reappear *in situ* on cooling and in the original form. There was no liquid crystal formation. Similar crystals with like behaviour were observed in ether extracts of the tissue. They appeared therefore to be ether-soluble and of fatty nature. Frozen sections and ether extraction of normal subcutaneous tissue revealed that there were present similar doubly-refracting crystals with like solubilities but a lower melting point. Experiments with pure tripalmitin showed that, on slow crystallisation from

ether at 0°, tufts of anisotropic, acicular crystals could be obtained, which, as regards solubility and melting point behaved similarly to the crystals from subcutaneous tissue. Further consideration of the nature of these crystals is made later, but it is to be noted that the assumption is generally made by histologists that anisotropic, acicular crystals are cholesteryl esters; the possibility of their being neutral fats has scarcely been considered. As these crystals were obtainable in the ether extract, it was decided to make a study of the ether extract in cases of sclerema and in normal cases with a view to attempting to determine (*a*) the nature of the crystals, (*b*) the reasons for the fat in the subcutaneous tissue in cases of sclerema having a melting point well above that of the body fat.

Chemical Investigation of Subcutaneous Tissues.

The ether extract of the tissue.

Although the first two cases were not studied chemically in any detail, they suggested the procedure which was adopted in the later ones. In these two cases the fat was melted out of the portions of the subcutaneous tissue and dried. Determinations of melting point, iodine value (Hübl), and free and total cholesterol were made by Dr J. W. McNee and similar treatment was given to the fat from two control cases (Table I).

In the remainder of the work (Tables II and V), the tissue was exhaustively extracted with ether, after sufficient grinding with sand and anhydrous sodium sulphate to produce a fine powder. Every precaution was taken to ensure that no oxidation of the extracted material should occur during the process of removal of the solvent and drying. As many determinations of the fat constants, etc., were made as was possible. The limiting factor in all cases was the small amount of material available, which in no case exceeded 7·25 g. As the melting point is of some importance, as the later results will show, it is necessary to state the exact procedure adopted in its determination. The fat, after being sucked up into an open capillary tube, was allowed to stand for 24 hours on ice; the melting point was taken as that temperature at which the fat began to rise in the open capillary; the determination was carried out at least twice on each sample of fat.

The results are given in Tables I and II. The unsaturated acid has been calculated from the iodine value, the assumption being made that oleic acid is the only unsaturated acid present. This would appear to be justifiable in control cases, but may not be entirely so in cases of sclerema, because traces of a precipitate were obtained on carrying out the bromide test. Owing to the very small amount of material available, it was not possible to prove definitely that this precipitate was due to the presence of acids more unsaturated than oleic, and even if it were, the relative amount in which they were present allows their effect on the iodine value to be neglected without introducing any considerable error.

Table I.

	Age (weeks)	Melting point ° C.	Iodine value (Hübl)	Free cholesterol %	Ester cholesterol %
Case 1	6	50·5	42·1	0·25	0·20
„ 2	12	38·5	52·0	—	—
Control 1	52	33·5	45·7	trace	0·82
„ 2	9	32·5	57·5	—	—

Table II.

Determination	Case 3 Biopsy material (age: 21 weeks)	Case 3 Autopsy material (age: 24 weeks)	Case 4 Autopsy material (age: 12 weeks)
Total cholesterol % (digitonin)	1·5	6·0	2·1
Unsaponifiable matter	—	13·6	3·1
Melting point ° C.	38·5	42·9	47·3
Acid value	5·4	9·0	5·9
Saponification value	211	206	195
Iodine value	52·3	54·0	45·5
Olein % (calc.)	60·8	62·7	52·8
Separated fatty acids.			
M.P. ° C.	—	45·1	49·7
Neutralisation value	—	216	206
Mean molecular weight	—	260	272
Iodine value	—	48·9	46·7
Oleic acid % (calc.)	—	54·3	51·8

Remarks.

The results obtained with the ether extract from the biopsy material in case 3 are not more than approximate because only 0·5 g. of ether extract was obtained; the corresponding autopsy material weighed 5 g. and that from case 4, 7·25 g.

There is a marked difference in the total cholesterol content of the autopsy and biopsy material in case 3. The patient was very emaciated at death, but there was no emaciation at the time of biopsy. It would seem probable that during this interval of 3 weeks, while fat was being absorbed from the subcutaneous depôts at a rapid rate, cholesterol was being absorbed much more slowly. This hypothesis would seem to receive some confirmation from the fact that there was a similar difference in the calcium content of the ether-extracted residue of the biopsy and autopsy materials. It would seem reasonable to suggest therefore that cholesterol and calcium were absorbed much more slowly, if at all, during the period when emaciation was taking place.

As will be seen from a comparison with the melting points, given later in the paper, for subcutaneous fat of normal children, the melting points of the sclerematous fat seem to be raised to a definite if limited extent. As the amount of cholesterol present was much above normal, it seemed of interest to see whether the addition of small amounts of cholesterol to normal fat would affect the melting point of that fat to any appreciable extent. Accordingly to normal fat (M.P. 28·4°) was added 2 % of cholesterol; the melting

point of the resultant mixture was 28·5°, the addition having raised the melting point by 0·1° only. Additions of tripalmitin to normal fat caused, however, marked elevations in the melting point as will be seen from the following table:

Table III.

	Added palmitin as % of resultant mixture	Average M.P. ° C.	Elevation of M.P. ° C.
Control fat	—	27·0	—
Control fat + palmitin	3·0	35·9	8·9
,, ,,	7·1	44·0	17·0
,, ,,	28·4	53·7	26·7

Further reference will be made to these results later.

The ether-extracted residue from sclerematous tissue.

It was of interest to enquire into the reasons for the opacity of the subcutaneous tissue which X-ray photographs had demonstrated. Estimations of calcium and phosphorus in the ether-extracted residue gave the following results:

	Case 3		Case 4	Control
	Biopsy	Autopsy	Autopsy	case
Ca as % dry residue	0·26	2·62	0·25	0·07
P (total) dry residue	—	1·41	0·47	—
Ratio Ca/P in dry residue	—	1·85	0·54	—

Ratio Ca/P for $Ca_3(PO_4)_2$ is 1·965.

There is a definite excess of calcium in the tissue in sclerema cases. Calcium is generally deposited in pathological tissues as phosphate or carbonate. In these cases there was a relative excess of phosphorus. Whilst, therefore, there is no evidence as to the form in which the calcium was actually combined, there is nothing in the figures contrary to the assumption that it was in the form of phosphates.

Chemical Constants of the Subcutaneous Fat of Normal Children.

It was hoped that a comparison of the results obtained by us with the fat of sclerematous patients and with normal subcutaneous fat, would throw some light on the abnormalities of the former, and provide an explanation of its occurrence and nature. The literature however contains but little information concerning subcutaneous fat of normal children. Such as there is concerns almost exclusively the degree of unsaturation determined by the iodine value or by the lead salt-ether method, and the melting point. Thus Langer [1881] noted the difference in melting point and consistency of the subcutaneous fat of infants and that of adults; he obtained 31 % solid fatty acids in the former and 10 % from the latter. Raudnitz [1888] found the melting point of the subcutaneous fat from an infant of 2 days and a child of 2 years to be 43·6° and 29·4° respectively. These are averages of the figures he obtained for the fat from various regions.

Knöpfelmacher [1897] found that the iodine value of the subcutaneous fat of a new-born child was lower than that of an adult. He determined the melting points and iodine values of the fatty acids obtained from seven children varying in age from 0–17 months. The melting points on the whole decreased with increase in age (from 48° to 37°), and the iodine values showed a progressive rise from 40·1 to 64·5. As a result of this work he made the interesting suggestion that the percentage of triolein in infant body fat is at its lowest value at birth and increases until the twelfth month when it reaches "adult" level.

Thiemich [1898] and Siegert [1902] also observed the smaller triolein content of child fat as compared with adult fat; the former was however unable to confirm Knöpfelmacher's hypothesis that the iodine values increased *regularly* to a maximum at the age of 12 months. Siegert suggested that the increase in the iodine values was due to a change from a milk to a mixed diet at the age of from 8 to 12 months. The following table contains the results obtained with the fat of non-sclerematous cases by Knöpfelmacher [1897], Thiemich [1898], Siegert [1902], Jaeckle [1902], Bardisian [1921], Finkelstein and Sommerfeld [1923] and Lasch [1925], and will serve as a rapid survey of the literature on this point:

Table IV. *Iodine values of the separated fatty acids according to age.*

Age	No. of cases	Iodine values		
		Lowest	Highest	Average
Premature	2	46·8	49·4	48·1
Newborn	16	36·9	49·2	42·2
1 day–1 month	7	38·1	60·7	47·1
1– 2 months	5	38·5	51·4	46·7
2– 3 „	6	41·5	58·7	50·1
3– 4 „	9	41·4	75·5	57·4
4– 5 „	4	40·6	47·8	44·3
5– 6 „	6	47·0	53·3	50·9
6– 8 „	7	46·1	56·2	49·6
8–10 „	3	51·7	61·6	57·0
10–12 „	4	55·5	63·9	61·0
Adult	—	—	—	65·0

NOTE. In those cases where the authors have made their determinations of the iodine values on the fat itself, the approximate corresponding values for the fatty acids have been calculated and have been included in the above table.

There is general agreement in the literature that the melting point of the fat of young infants is higher than that of adults and decreases in general with increase in age up to about one year. The iodine values as will be seen from Table IV behave in the reverse sense (iodine values from first to eighth month, 37–55, increasing rapidly to "adult" level, 60–65).

The only recorded saponification values are those of Jaeckle [1902], 204 for two infants of under one month and 193·3 to 199·9 as the extremes in seven adult cases; Bardisian [1921] gives 212–228 for children aged 3 months to 5½ years. Unna and Golodetz [1909] give the free cholesterol as 0·14 % and that as ester 0·04 %, total 0·18 %.

As the bulk of the work on normal subcutaneous fat was carried out prior to 1902, and as there are but few recorded figures to serve as a basis for comparison with those obtained from the fat of sclerematous patients, it was deemed wise to make a study of subcutaneous fat of normal infants. This was obtained by the method already described for sclerematous fat, and the results are given in Table V.

Table V. *Results of examination of "ether extract" from a series of control cases (autopsy material throughout).*

Case	a	b	c	d	e	f	g	h	i
Age	3/12	4/12	8/12	10/12	10/12	1 year	14/12	2 years 10 months	40 years
Cause of death	Acute gastro-enteritis	Gastro-enteritis	Influenzal menin-gitis	Abscess of neck, and septi-caemia	Con-genital heart disease	Osteo-myelitis Purulent arthritis Gastro-enteritis	Genera-lised tuber-culosis	Osteo-myelitis	Placenta praevia
Total cholesterol % by colorimetric method	—	—	—	0·19	—	—	—	—	0·26
M.P. of ether extract, °C.	37·3	30·5	27·9	29·4	32·7	26·9	29·0	21·5	27·3
Acid value	2·0	1·7	1·2	0·6	2·7	0·9	0·9	Nil	Nil
Saponification value	206	203·6	199·7	203	203·3	201·8	198·3	199·4	199·0
Iodine value	53·8	56·9	60·6	56·6	57·4	58·4	61·0	67·5	65·0
Calculated olein %	62·5	66·1	70·4	65·7	66·8	67·8	70·8	78·3	75·5
Separated fatty acids:									
M.P. ° C.	—	38·6	36·1	—	39·9	37·8	35·8	34·0	33·5
Neutralisation value	—	214·5	209·6	—	209·7	209·2	206·5	206·6	207·0
Mean molecular weight	—	261·8	267·7	—	267·5	268·1	271·8	271·5	271·0
Iodine value	—	59·6	62·4	—	59·8	61·1	62·9	70·3	65·7
Calculated oleic acid %	—	66·2	69·3	—	66·5	67·9	69·9	78·1	73·0

These figures confirm the observations of other workers that the iodine value and melting point of the fat increase and decrease respectively from birth to about 12 months of age but without complete regularity. It is of interest to notice that there is however little variation in the saponification value which appears to approximate closely to 200, and to vary to a smaller extent than the iodine value. The obvious objection to these results is that they have been obtained from pathological cases, but it is virtually impossible to obtain post-mortem specimens from healthy infants, and the results do not contain any figures which appear so abnormal as to warrant their being viewed with suspicion.

With these figures for comparison, we may now consider the various hypotheses which have been put forward by various workers to account for the appearance of the exceptionally solid fat in the subcutaneous tissues of infants suffering from sclerema.

There are 18 cases of sclerema neonatorum in which chemical analyses have been reported in the literature, and the various theories put forward may be briefly reviewed. Langer [1881] suggested that in diseases of infants preferential oxidation of olein might occur, the resulting increase in the saturated acids and a lowering of the body temperature being responsible for the hardening of the fat. Knöpfelmacher [1897] believed that sclerema was due to a combination of three causes: loss of fluidity of the subcutaneous

fat, lowering of body temperature, and the high temperature of solidification of the fat of infants. Siegert [1902] pointed out that the diminished olein hypothesis could not hold in view of the observations of Thiemich [1898] that there was no significant difference between the iodine values of the fat of two sclerematous infants and that of normal infants of the same age, and that it was mere assumption that olein was more readily oxidised than the saturated acids. Miura [1905] concluded that "the fat of scleremic infants did not differ materially from the fat of normal infants of the same age," and Sarvonat [1906] that "neither the body temperature nor the oleic acid content would explain the hardening of the tissues." Bayer [1908] maintained that a pathological reduction of the oleic acid was the cause of sclerema, and lastly Finkelstein and Sommerfeld [1923], as a result of work on five cases, suggested that the disease was due to some change in the colloids of the subcutaneous tissue.

Langer's hypothesis is obviously untenable and need not be discussed. The only other hypothesis is that founded on the observation that the melting point of the fat in sclerematous children is above normal (Knöpfelmacher, Bayer).

In every one of the five analyses made by us the melting point has been found to be greater than that for the fat from non-sclerematous children of the same age. Is this raised melting point due to a decrease in the amount of oleic acid present? Our results are set out in Table VI.

Table VI.

Age (weeks)	Iodine values		Melting points ° C.	
	Sclerema	Normal	Sclerema	Normal
6	42·1	—	50·5	—
12	52·0	53·8	38·5	37·3
12	45·5	53·8	47·3	37·3
21	52·3	56·9–60·6	38·5	30·5–27·9
24	54·0	56·9–60·6	42·9	30·5–27·9

In view of the fact that the iodine values in the control cases vary among themselves, it will be seen that the above table provides no great evidence in favour of the diminished olein hypothesis, for there are no significant differences in the iodine values of the sclerematous and normal cases, although the former tend to lower values. This however does not disprove the hypothesis, for but small alterations in the amount of olein in a fat have a marked effect on the melting point without affecting the iodine value to any appreciable extent (the addition of 3 % of palmitin to a normal fat raised the melting point by 8·9°; see Table III). Further if 3 % of olein present in a fat of iodine value 65 and saponification value 200 were replaced by tripalmitin, the iodine value of the resulting fat would be 62·4 and its saponification value 200·5, differences of 2·6 and 0·5 respectively. The fat would then have a definitely raised melting point, but the differences between the two constants men-

tioned would not be detected because those of normal infants' fat vary among themselves over a much greater range than this.

The literature records no serious attempt to determine the nature of the anisotropic crystals referred to earlier in this paper, although crystals have been observed by Knöpfelmacher [1897] and Lieberthal [1918]. It would appear probable that they are similar to those observed in normal subcutaneous fat, and in that case they are presumably tripalmitin or tristearin. Their solubility excludes the possibility of their being soaps or urates; nor can they be fatty acids on account of the low acid value of the fat and their behaviour towards alkaline alcohol. It is improbable that they are cholesteryl esters because (*a*) microchemical tests for cholesterol were negative; (*b*) on careful warming under the polarising microscope and cooling, no liquid crystal formation was ever observed; (*c*) the melting point of the sclerema crystals was lower than that of true cholesteryl esters as seen in frozen sections of cutaneous xanthoma; (*d*) the actual amount of cholesterol present was very much less than would have been expected from the microscopic appearance of the sections.

The solubility of the sclerema crystals in acetone, and the fact that on ashing they left no residue, rule out the possibility of their being cerebrosides.

We conclude, therefore, that the crystals are probably neutral fats, either palmitin or stearin or both, or glycerides of the higher fatty acids. Such a hypothesis will explain the raised melting point and it will fit in well with the observation that fat from scleremateous patients has an iodine value which, while not differing markedly from that of normal subcutaneous fat, tends to a somewhat lower value.

Summary.

1. The analytical constants of the subcutaneous fat of eight infants and one adult have been determined. The iodine value is lowest at birth and increases to the adult value from the eighth to the twelfth month of life; there is a corresponding decrease of melting point with age. The saponification value is almost constant at 200.

2. Similar analyses have been made in four cases of sclerema neonatorum, in which the fat appears to have a higher melting point and a slightly lower iodine value than the corresponding values of the fat in infants of the same age.

3. The raised melting point is believed to be due to the presence of an excess of glycerides of the higher fatty acids—probably of palmitic acid—which causes a slight decrease in the oleic acid content of the fat.

4. Subcutaneous tissue in cases of sclerema contains an abnormal amount of cholesterol, calcium and phosphorus.

We wish to thank Dr J. W. McNee for permission to include his results in cases 1 and 2. A full account of the clinical, histological, X-ray findings, etc., will be published elsewhere by one of us (G.A.H.).

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XV. THE DETERMINATION OF IRON IN BLOOD, TISSUES AND URINE.

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Introduction.

Of all colorimetric methods used for the determination of small quantities of iron the thiocyanate method is the best known, and is one which, provided its limitations are well understood, gives very good results. An extremely useful modification was introduced by Marriott and Wolf [1906] which consists in the development of the thiocyanate colour in the presence of acetone. By this means not only is the colour intensified and stabilised, but in our experience, the effect of interfering substances is considerably minimised and in some cases entirely prevented. Walker [1925], who in other respects has published a useful study of the thiocyanate method, appears to have overlooked this important modification. Wong [1923] omits its use in the method he describes on account of possible changes in the volume of the final solution due to the volatility of the acetone. This objection is of no practical importance provided the final solution is made up in a stoppered graduated flask, for, since the final solution contains 50 % of water with considerable quantities of ammonium thiocyanate in solution, the vapour pressure will be considerably lower than that of pure acetone; and as the solution has a free surface exposed to the air only during the actual comparison in the colorimeter, any change in the concentration of the solution due to evaporation of acetone can have no appreciable effect on the final result. The advantages to be derived from the use of acetone considerably outweigh any objections to its use, particularly in the case of tissues and urine, where the intensification of the colour is highly desirable, and where interfering substances, notably phosphates, are present in relatively large quantities.

The Determination of Iron in Blood.

Lapicque [1889] appears to have been the first to use the thiocyanate method for this purpose. Berman [1918] used concentrated hydrobromic acid to split off the iron held in combination in blood and oxidised it to the ferric condition, at the same time destroying organic matter, with potassium permanganate. Wong carried out oxidations by means of sodium or potassium chlorate.

In the present method we have largely followed Wong's procedure, but have introduced the use of "perhydrol" (100-volume hydrogen peroxide) in place of chlorate and utilised the acetone modification as described by Berman. "Perhydrol" has two advantages over chlorate, namely, that it leaves no residue, and that the steady evolution of oxygen which results from its addition ensures steady ebullition, thus avoiding the possibility of loss by spurting which we have found to be a real danger in Wong's method.

Method.

1 cc. blood is measured into a test tube containing 4 cc. of distilled water. After thorough mixing, 1 cc. of this diluted blood is transferred to a Pyrex test tube (200 × 25 mm.) followed by 1 cc. of concentrated sulphuric acid. The tube is clamped and held at an angle of 40° to the horizontal. The contents are boiled rather vigorously until practically all the water has been driven off and white fumes begin to be evolved. Heating is discontinued for about half a minute, after which time 0·5 cc. of "perhydrol" is added to the tube, drop by drop, from a teat pipette. Boiling is then repeated. A brisk evolution of oxygen occurs and the solution in the tube assumes an amber colour. When white fumes are again evolved heating is again discontinued and after cooling for half to one minute a further 0·5 cc. of "perhydrol" is added as before. Heating is resumed and the solution usually becomes colourless. If all colour has not disappeared a further 0·3 cc. of "perhydrol" is added. Heating is continued for one minute after the solution has become colourless. The solution is now allowed to cool completely, then it is diluted with about 5 cc. of distilled water and transferred to a 50 cc. stoppered graduated flask. Into another similar flask are placed 1 cc. of a standard iron solution containing 0·1 mg. iron per cc. and 1 cc. concentrated sulphuric acid. Water is added to both flasks up to a volume of about 18 cc. after which 25 cc. acetone are added. The contents of the flasks are thoroughly mixed and allowed to cool to room temperature. To each flask are then added 5 cc. of 3 *M* ammonium thiocyanate, the contents mixed and made up to the mark. The two solutions are then compared in a colorimeter.

If the standard is set at 20 mm., then

$$\frac{20}{R} \times 50 = \text{mg. iron per 100 cc. blood,}$$

or $\frac{20 \times 50}{R \times 3{\cdot}35}$ = haemoglobin in blood (haemoglobin contains 0·335 % of iron),

where R is the colorimeter reading of the solution tested.

Preparation of standard iron solution.

Dissolve 0·7 g. pure ferrous ammonium sulphate in about 50 cc. of distilled water. Add to the solution 20 cc. 10 % iron-free sulphuric acid, warm slightly and then add 0·1 *N* (approx.) potassium permanganate solution to

oxidise the ferrous salt completely. Dilute with distilled water to 1 l. Each cc. contains 0·1 mg. iron.

The accuracy of the method was tested by adding to a specimen of blood, diluted to five times its original volume with distilled water, various quantities of the standard iron solution and then determining the total amount of iron present in each case. The concentration of the standard was varied so as to obtain colorimeter readings for the solutions tested lying between 15 and 25 when the standard was set at 20. The blood used was found to contain 47·5 mg. iron per 100 cc.

The following results were obtained:

Amount of iron added mg.	Total amount present mg.	Total amount recovered mg.
0·02	0·115	0·113
0·04	0·135	0·132
0·06	0·155	0·154
0·08	0·175	0·177
0·10	0·195	0·194

The Determination of Iron in Tissues.

For this purpose most methods require complete destruction of organic matter either by ignition or by oxidation with sulphuric and nitric acids.

The present method involves the partial destruction of organic matter with sulphuric acid followed by complete oxidation of remaining organic matter in an aliquot portion of the resulting fluid by "perhydrol." The iron is then determined by the thiocyanate method described above.

The presence of acetone has been found necessary in order to obtain satisfactory results as otherwise the presence of phosphates introduces an appreciable error.

Method.

The organ or tissue to be examined is minced and washed with water to remove blood. It is then dried in a steam oven. The dried material is then ground in a mortar until it passes through a 30-mesh sieve.

1 g. of this material is weighed into a 300 cc. Kjeldahl flask to which are added 10 cc. concentrated H_2SO_4. The flask is heated in a fume chamber, gently for 15 minutes, and then strongly for 30 minutes. At the end of this time the walls of the flask are free from charred material and its contents consist of a brown homogeneous fluid. This is allowed to cool and then diluted with an equal volume of water and transferred to a 50 cc. stoppered graduated flask. A rise of temperature occurs on diluting and washing the fluid into the graduated flask. When room temperature is again reached the contents of the flask are made up to the mark. A certain amount of light, flocculent precipitate forms on adding water to the acid digestion fluid. The contents of the flask are therefore thoroughly shaken and 5 cc. are withdrawn immediately and placed in a Pyrex tube, to which 1 cc. of "perhydrol" is

added. The tube is inclined as in the previous method and the oxidation with "perhydrol" there outlined is followed. If much iron is present the final solution has a slight yellow colour after oxidation which almost entirely disappears on cooling.

After cooling, the solution is transferred to a 50 cc. graduated flask and acetone and thiocyanate solution added as before, a standard for comparison being also prepared as already described.

In the case of organs containing more than the normal amount of iron (*e.g.* where siderosis is present) 0·5 g. should be taken instead of 1 g., and it may be found necessary to prepare a stronger standard solution for colorimetric comparison.

The following table indicates the accuracy with which known amounts of iron added to various tissues before the sulphuric acid digestion have been recovered by this method.

Tissue	Iron in tissue mg.	Iron added mg.	Total iron present mg.	Total iron recovered mg.
Kidney A, 0·25 g.	2·90	0·20	3·10	3·09
„ B, 1·0 g.	0·98	0·30	1·28	1·26
Liver B, 0·5 g.	1·30	0·40	1·70	1·68
„ A, 0·5 g.	0·60	0·50	1·10	1·105
Spleen A, 1·0 g.	0·66	0·60	1·26	1·30
„ B, 0·5 g.	0·63	0·70	1·33	1·29

The Determination of Iron in Urine.

The concentration of iron in urine is extremely small, amounting to about 1 mg. in the whole 24-hour specimen in the case of normal persons. Further, the presence of phosphates in considerable amounts introduces difficulties whatever method of analysis be employed.

In the present method partial destruction of organic matter is carried out as in the previous method. After the final complete oxidation by "perhydrol," strong heating is continued for at least 3 minutes until practically all sulphuric acid is driven off. Under these conditions orthophosphoric acid is changed to metaphosphoric acid, which appears to cause less interference with the colour development. The sulphuric acid driven off is replaced before the colour is developed as it is essential to have a sufficient quantity of sulphuric acid in order to get correct results. Marriott and Wolf showed that the interference of phosphates can be prevented by having a sufficient concentration of mineral acid.

Acetone is essential in the case of urine for under the conditions employed in the following method the colour produced by the thiocyanate is barely perceptible in its absence. Its presence however introduces a complication since it precipitates some of the inorganic salts which are present. The precipitation amounts to little more than a cloudiness which however is quite sufficient to make colorimetric comparison impossible. The precipitate is, however, readily removed by centrifugalisation.

The colour produced in this method fades much more rapidly than in the previous methods, hence it is necessary to lose no time in making the colorimetric comparison.

Still, though the method is more difficult than the one described for blood and tissues, it is simpler than the methods at present in use for urine, and provided careful attention is paid to all the details no fault will be found with the accuracy of the results obtained.

Method.

To 100 cc. of the 24-hour specimen of urine placed in a 300 cc. Kjeldahl flask, 10 cc. of concentrated sulphuric acid are added. The contents of the flask are vigorously boiled until frothing begins to take place. Heating is then carried on with caution until frothing ceases. A full flame is then used again until the walls of the flask are free from charred material and a homogeneous fluid results. After cooling, this fluid is diluted and transferred to a 50 cc. graduated flask whose contents are made up to the mark when they have cooled again to room temperature. 10 cc. of the liquid are transferred to a Pyrex tube and concentrated by boiling. Until the volume has been considerably reduced the flame of the burner must not be directed to the bottom of the tube, but to the wall of the tube just below the surface of the liquid. When nearly all the water has been driven off and white fumes begin to be evolved, 0·5 cc. perhydrol is added as before, followed by further quantities of 0·3 cc. and 0·2 cc. After the last addition the resulting liquid is boiled vigorously for 3 minutes, when practically no more fumes are evolved. The liquid is then allowed to cool, diluted with about 2 cc. distilled water and transferred to a 25 cc. stoppered graduated flask. 1 cc. concentrated sulphuric acid is added and 12·5 cc. acetone. The contents of the flask are made up to about 20 cc. and allowed to cool to room temperature. 3 cc. of the thiocyanate solution are then added and the contents of the flask well mixed and made up to the mark. The standard for comparison is prepared at the same time in a similar flask using 1 cc. of a solution containing 0·01 mg. iron per cc., 1 cc. concentrated sulphuric acid, and 12·5 cc. acetone. The unknown solution as thus prepared is cloudy. A portion of it is therefore immediately centrifuged in a closed tube for 5 minutes, when the clear supernatant liquid which results is compared with the standard in the colorimeter.

The iron solution used for preparing the standard for comparison is one-tenth the concentration of the standard previously used and is prepared from the latter by suitable dilution with distilled water.

It is essential in this method, where very small quantities of iron are estimated, to prevent dust from falling into the solutions, as it has been found that the atmospheric dust, at any rate in an industrial centre, contains sufficient iron to cause errors in the result if precautions are not taken to exclude it.

The following table shows the agreement obtained between the results of two separate determinations on the same specimen, and also shows the accuracy with which known quantities of iron added to urine, before the acid digestion, may be recovered by this method.

Iron in urine mg. %	Iron added mg.	Total iron present mg.	Total iron recovered mg.
0·043	—	—	—
0·044	—	—	—
0·0435	0·05	0·0935	0·092
,,	0·10	0·1435	0·145
,,	0·15	0·1935	0·186
,,	0·20	0·2435	0·250

SUMMARY.

Methods are described for determining the amount of iron present in blood, tissues and urine using the colorimetric method dependent on the formation of ferric thiocyanate in presence of acetone. It is shown that these methods are capable of a high degree of accuracy. They are simpler and less time-consuming than the methods at present in use.

These methods are being employed in a quantitative study of certain aspects of iron metabolism.

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XVI. NOTE ON THE EXCRETION OF PHOSPHATE DURING WATER DIURESIS.

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(*Received December 3rd, 1925.*)

In the course of some experiments on exercise we had occasion to examine the excretion of inorganic phosphate during a copious diuresis—500 to 1500 cc. per hour—produced by drinking water. The consistent results obtained have an important bearing on several current theories of phosphate excretion.

Details of sampling and analysis are given in a previous paper [Havard and Reay, 1925]. The whole blood phosphate figures are assumed in this discussion to be identical with those for plasma [Zucker and Gutman, 1922].

Table I.

Time a.m.	Urine cc.	Water rate cc. per hour	P (mg.) per 100 cc.	P (mg.) per hour	P (mg.) per 100 cc. blood	Urea (g.) per litre	Urea (g.) per hour	Sulphates (mg. SO_4'') per 100 cc.	Sulphates (mg. SO_4'') per hour
Exp. 1. A.C.									
A. 9.30–10.20	140	168	10·40	17·5	10.55 a.m. 3·60				
B. 10.20–10.45	355	852	1·46	12·5	11.20 a.m. 3·67				
C. 10.45–11.20	680	1168	1·14	13·3	—				
D. 11.20–11.35	663	652	1·70	11·1	—				
Exp. 2. H.K.B.O.									
A. 9.50–10.21	202	391	2·7	10·6	—	4·25	1·66	—	—
B. 10.21–10.47	524	1210	0·733	8·9	10.45 a.m. 2·97	0·90	1·09	7·82	94·6
C. 10.47–11.11	518	1270	0·840	10·6	11.0 a.m. 2·97	0·95	1·20	7·61	96·6
						Average blood-urea value 0·3 g. per litre		Average blood-sulphate value 2·3 mg. SO_4'' %	

Table I gives figures for two typical experiments taken from a series of fifteen. Some more figures are given in the previous paper. In Exp. 1, showing the onset of diuresis, the kidney is at first concentrating phosphate from 3·6 mg. P per 100 cc. plasma to 10·4 mg. P per 100 cc. urine. As the diuresis develops, the kidney dilutes phosphate first to 1·46 mg. P per 100 cc. and later to 1·14 mg. P per 100 cc., whilst sulphates and urea are being concentrated. This fall in urinary P below plasma level has been noted in 44 different samples of urine.

Yet all the while the rate of phosphate excretion is comparatively constant, and actually falls, as the diuresis develops, from 17·5 to 12·5 mg. P per hour. In fact the figures for the rate of phosphate excretion are even more accurately constant during a heavy diuresis, when phosphate is being greatly diluted below the plasma value, than during a moderate diuresis when dilution is less or even absent.

In Exp. 2 it is well seen that the greater the diuresis, the greater is the dilution, so that the resulting rate of excretion is very accurately constant.

From these data, then, we may conclude that, given a constant plasma phosphate, and in the absence of disturbing factors such as acidosis and alkalosis, the kidney in excreting phosphate can maintain a constant rate of excretion quite independent of whether in so doing it has to concentrate or dilute the phosphate.

This result is not reconcilable with the theory that the urine is produced by glomerular filtration and reabsorption, as recently stated by Mayrs [1922], unless phosphate is considered to be a "threshold" substance. Mayrs holds that "absorption in the case of 'no-threshold' bodies is negligible, and may be regarded merely as evidence of renal inefficiency." This is obviously inconsistent with the very accurate regulation of the urinary phosphate percentage below the plasma value, while sulphates and urea are still being concentrated. By no process of merely passive "leaking back" could the percentage of phosphate in the glomerular filtrate be reduced 50 % below that of the plasma. At least, if this were anything but a definitely regulated activity of the kidney, we should expect that the greater the speed at which the glomerular filtrate traverses the tubules the nearer would the percentage of phosphate approach the plasma value. In fact, the reverse is found to be the case and the phosphate percentage falls even lower as the diuresis increases [cf. White, 1925]. It seems, then, that on the reabsorption theory phosphate must leave the category of "no-threshold" bodies along with urea [Mayrs, 1922], ammonia and creatinine [Marshall and Crane, 1924], which leaves only sulphates and foreign bodies. It has previously been suggested by Wigglesworth and Woodrow [1924] that there is probably a threshold value for plasma phosphate at about 2·4 mg. %.

Another possible explanation is suggested in a paper by Eichholtz and Starling [1925]. They conclude from perfusion experiments on the isolated kidney that a great part of the phosphates present in normal plasma is present as a non-ionised colloidal calcium compound to which the glomerular membrane is normally impermeable. "If this be true," they continue, "the secretion of phosphates in normal urine must be due to processes of excretion by the tubular cells." These conclusions are supported by work by Barkus [1924] on the excretion of parenterally introduced phosphates in sheep, and by work by Addis, Meyers and Bayer [1924], on anaesthetised rabbits; Bock and Iversen [1921] as a result of experiments on water diuresis and purine diuresis, came to the conclusion that phosphate is excreted by the tubules.

Our own results fit in most easily with this conception of phosphate excretion. If the glomerular membrane is normally impermeable to phosphates and the rate of excretion is governed solely by the processes of secretion by the tubule cells, then the rate of excretion will naturally be quite independent of any change in the flow of water through the glomerular membrane.

The main difficulty in accepting this theory unreservedly is in the statement by Cushny [1919] that the phosphate in the blood is freely filterable through a collodion membrane. Eichholtz and Starling, however, found that cyanide made the glomerular membrane permeable to the colloidal phosphate before the proteins, so possibly the particles may pass a collodion membrane.

Our results are not inconsistent with the idea of a glomerular filtrate being modified as it passes down the tubules by reabsorption of phosphate or secretion of water. Certainly it is difficult to picture clearly the regulation of the processes in the tubule cells in turning over from concentrating to diluting the glomerular filtrate as the diuresis increases, while maintaining accurately a constant rate of excretion. Nevertheless, this sometimes happens in the case of chlorides, and these are almost universally considered to be filtered through the glomerulus. On the whole, however, our results are more easily accounted for by the idea suggested by Eichholtz and Starling than by any previous theory.

Summary.

1. The rate of phosphate excretion is independent of the water rate even when, owing to copious diuresis, the urinary phosphate is below the level of the plasma phosphate.

2. It is shown that this is inconsistent with the view that phosphate is a "no-threshold" substance as represented in Cushny's theory of kidney secretion.

3. The bearing of this result on other theories of kidney secretion is briefly discussed.

We wish to thank Mr J. B. S. Haldane for bringing to our notice the importance of these results and also for much helpful criticism and advice.

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XVII. A BUFFER MIXTURE FOR THE ALKALINE RANGE OF HYDROGEN ION CONCENTRATION DETERMINATIONS.

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(*Received December 23rd, 1925.*)

The preparation of carbonate-free sodium hydroxide, as required for the Sörensen and Clark and Lubs buffer mixtures is undoubtedly troublesome, and neglect of the proper precautions gives rise to appreciable errors in the resulting buffer solutions. In the course of a biological research it was necessary to leave an alkaline solution, the alkalinity being due to sodium hydroxide, exposed to the air, with the natural result that carbon dioxide was absorbed and the alkalinity reduced. It was thought possible that were sodium carbonate used, instead of sodium hydroxide, the preparation of fresh solutions would be facilitated and the error due to absorption lessened. Were such a carbonate mixture however to be used much below p_H 8 it would tend to become more, and not less, alkaline, this being due to the solution giving off more carbon dioxide than it absorbs, the partial pressure of this constituent having risen.

Another point had to be considered, namely, whether the sweeping out of carbon dioxide during the bubbling of hydrogen through the Clark [1922] electrode vessel used in standardisation might not be a source of error. Owing, however, to the fact that the carbonate solution used was concentrated enough to be highly buffered no difficulty was experienced on this score.

The following solutions are accordingly recommended for use from p_H 7·8–10·8, though they cover a slightly more extended range:—(*a*) 0·2 *M* boric acid made 0·2 *M* with respect to potassium chloride also, as recommended by Clark [1922]. One litre should contain 12·4048 g. boric acid (taking the atomic weight of boron as 11·0, not 10·9 the more recent value) and 14·912 g. potassium chloride. (*b*) 0·2 *M* sodium carbonate 21·2000 g. per litre, prepared anhydrous from the bicarbonate as is usual in alkalimetry. To make up the mixtures for use the quantities of (*a*) and (*b*) shown in the table are mixed and diluted with 100 cc. water.

The first three columns of the table record the p_H values found for various mixtures of the two solutions in multiples of 5 cc. The last three columns show the volumes of the two solutions to be mixed to give p_H values differing

by 0·2; the volumes were read off from a graph, which has been omitted to economise space. These buffer solutions keep well if stored in waxed bottles tightly stoppered.

Water 100 cc. and			Water 100 cc. and		
Boric solution cc.	Carbonate solution cc.	p_H at 16°	Boric solution cc.	Carbonate solution cc.	p_H at 16°
95	5	7·44	93·8	6·2	7·6
90	10	7·93	91·7	8·3	7·8
80	20	8·43	88·8	11·2	8·0
70	30	8·78	85·0	15·0	8·2
60	40	9·09	80·7	19·3	8·4
50	50	9·39	75·7	24·3	8·6
45	55	9·53	69·5	30·5	8·8
40	60	9·69	63·0	37·0	9·0
30	70	9·98	56·4	43·6	9·2
20	80	10·25	49·7	50·3	9·4
10	90	10·59	42·9	57·1	9·6
5	95	10·85	36·0	64·0	9·8
—	—	—	29·1	70·9	10·0
—	—	—	22·1	77·9	10·2
—	—	—	15·4	84·6	10·4
—	—	—	9·8	90·2	10·6
—	—	—	5·7	94·3	10·8
—	—	—	3·5	96·5	11·0

When working at p_H values above 9·6 difficulties are experienced in the choice of indicators; below that, thymol blue and xylenol blue are both satisfactory and stable, but the only sulphonephthalein recorded by Clark as covering a markedly higher range is nitrothymolsulphonephthalein, which changes from violet to yellow between p_H 9·2–11·5; it, however, does not appear to be obtainable at present. Phenolphthalein may, however, be used up to p_H 10·5, and we have found the indicator now sold by the British Drug Houses, Ltd., as alizarin yellow G useful beyond this, the range being given as from p_H 10·1–12·1. According to Clark's list of synonyms this is identical with Michaelis' alizarin yellow GG, which is *m*-nitrobenzene-azo-salicylic acid, and changes from almost colourless to yellow. The alizarin G supplied to us, however, changes from pale yellow to orange and is Sörensen's alizarin yellow R, which is the corresponding *para* compound. In future it will be described as the latter. Thymolphthalein, which gives very beautiful blue shades between p_H 9·3–10·5, unfortunately fades rapidly.

Experimental.

The determinations were made with a Clark electrode vessel and shaking apparatus and a Leeds and Northrup potentiometer. The hydrogen used was generated electrolytically and traces of oxygen removed by passing over platinised asbestos in a hot tube. The two electrodes and connections were protected from air currents by a wooden box, but the temperature taken was that of the liquid in the hydrogen electrode vessel; the mean temperature of the twelve readings was 15·6°, but each reading was corrected to 16°, assuming that the coefficient is such that a rise of 6° results in reducing the

p_H value by 0·10, as for pure water; the assumption is certainly not accurate but appears to be sufficiently near for the purpose required and the results were found to lie well upon a curve.

The values given are for one atmosphere pressure of pure hydrogen at 0°; they have been corrected for the pressure of water vapour and for temperature. The potassium chloride used was of analytical reagent quality once recrystallised from glass-distilled water.

The standard cell was taken to give 1·0185 volts at 16° and the potentiometer was set accordingly. The value taken for the $N/10$ calomel electrode was 0·3381 volt at 16°, obtained by extrapolation from Clark's value 0·3380 at 18°. In making up the electrode re-distilled mercury and specially prepared calomel, from the Cambridge Instrument Co., Ltd., were used.

In order to test the accuracy of the working, $M/20$ acid potassium phthalate was prepared by resubliming phthalic anhydride and half neutralising. This was twice recrystallised and dried at 110°. By extrapolation from 18°, Clark's values for this solution were 0·5663 volt at 16·5° and 0·5657 volt at 15·4°, whereas those found by us were 0·5655 and 0·5664 respectively, differences of $-0·0008$ and $+0·0007$. Closer agreement is desirable, and the differences are probably due to inadequate temperature control, as the calomel electrode was not necessarily at the same temperature as the hydrogen electrode. The 0·8 millivolt difference however corresponds in this region to only p_H 0·013 and the value for $M/20$ acid potassium phthalate at 15·4° is p_H 3·996. In the region of p_H 10 a difference of 0·8 mv. corresponds to slightly under p_H 0·01. Since the differences between our values and Clark's for the 0·05 M acid potassium phthalate solution almost vanish when the two results are averaged, it is clear that no systematic error impairs the accuracy of these results.

It may be added that the inclusion of a variable resistance rheostat of 225 ohms, in addition to the fixed resistance supplied in the circuit of the rocking apparatus, was found to be essential.

The authors desire to acknowledge their indebtedness to the Department of Scientific and Industrial Research for a grant for the purchase of the potentiometer outfit and to the Government Grant Committee of the Royal Society for a further grant for working expenses.

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XVIII. NOTE ON ANAEMIA, UROBILINURIA AND INTESTINAL HAEMORRHAGE IN RABBITS IN CONSEQUENCE OF EXCLUSIVE NUTRITION WITH COW'S AND GOAT'S MILK.

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(*Received December 20th, 1925.*)

During many years, physicians believed that the anaemia which develops if men or animals are exclusively fed with milk is caused by lack of iron, in consequence of which the formation of haemoglobin would become insufficient (*milk-anaemia by decreased formation*).

Afterwards [Czerny, 1912; Kleinschmidt, 1916] it was recognised, that in addition to the forms of anaemia just mentioned there are cases in which the clinical picture proves an increased destruction of red blood corpuscles. Here the organism makes strong but unsuccessful efforts to compensate for the increased loss of haemoglobin, so that the highest degrees of anaemia may be observed (*milk-anaemia by increased destruction*).

Now it has been noted in Holland, Germany, Austria and Switzerland, that in infants under one year old exclusive feeding with goat's milk is more frequently followed by severe anaemia than exclusive feeding with cow's milk [Scheltema, 1916; Korteweg, 1916; Brouwer, 1922, 1923, 1925]. Particularly goat's milk gives rise to those types of anaemia in which the symptoms of increased destruction and formation of erythrocytes are apparent [Brouwer, 1922, 1923].

The direct causes of these "milk-anaemias by increased destruction" are unknown. Probably they would be discovered if one succeeded in provoking these milk-anaemias in experimental animals. The experiments here to be described were intended to investigate this point in young rabbits, especially with a view to the goat's milk-anaemia of infants.

It must however be mentioned that, though we succeeded in causing severe anaemia, the symptoms which occur so frequently in anaemia by goat's milk (polychromasia, basophile granulation, normoblastosis, megaloblastosis) were not found. In the stained blood film preparations (Pappenheim) only a moderate anisocytosis and a slight anisochromia could be seen. After the death of the animals, we found with the aid of the method of Tirmann-

Schmelzer-Hueck only very little iron-containing pigment in the spleen and in the liver. Moreover, there was no apparent difference between the consequences of feeding cow's milk or goat's milk.

Therefore it remains uncertain whether experiments with rabbits are suitable to contribute to the solution of problems concerning anaemia caused by goat's milk. Perhaps some other animal, *e.g.* the dog, would be better. In 1924 Beumer and Wieczorek [1924] believed that they had shown that in this animal feeding with goat's milk leads much sooner to a severe anaemia than feeding with cow's milk.

Nevertheless, our investigation has been interesting because some symptoms were noted which indicate, that after all in our experiments the anaemia is totally or at least partially caused by an increased loss of erythrocytes.

For this research four young rabbits were taken from two litters (age three to four weeks), and of these in both cases two were fed with raw cow's milk and two with raw goat's milk. Moreover, wood shavings were given in the first experiment; they were omitted in the second experiment.

The cages were provided with screen bottoms of galvanised iron gauze to prevent access to faeces. Unfortunately, notwithstanding the protecting layer, the iron wire rusted somewhat, so that the possibility that small amounts of this metal were ingested cannot be excluded.

The cow's milk was derived from a herd of several animals; the goat's milk from one single animal. The fat percentage of the cow's milk was 2·9 to 3·3; that of the goat's milk 5 to 6·5.

The experiment with the first four animals was made in winter, when the lactating animals were receiving a winter ration; the experiment with the second four in summer, when the cows and goat ate as much grass as they liked.

In five of these eight cases a severe anaemia developed in the course of some weeks to some months. In this lapse of time the haemoglobin content declined to ± 25 (Sahli) and the number of red blood corpuscles below three millions. This anaemia was accompanied by slight, moderate or even severe urobilinuria[1]. All the animals died after one to several months, and the same occurred with the animals in which only a slight anaemia or no anaemia at all developed.

In the animals fed with cow's milk, death occurred in the first experiment after 46[2] and 53 days and in the second after 58 and 105 days; and in the animals fed with goat's milk respectively after 21, 82, 73 and 80 days.

[1] As liver and spleen contained very little haematosiderin, the direct mutual dependence of anaemia and urobilinuria could not be proved. *A priori* the possibility that abnormal liver-function may be the cause of the urobilinuria cannot be denied. Indeed, by microscopical examination some fatty degeneration in the centres of the liver lobules and in the layer immediately underneath the liver capsule was unmistakable. It seems however improbable that this explains the urobilinuria.

[2] In the rabbit that lived 46 days, intussusception of the small intestine was found *post mortem*, probably the direct cause of death.

Loss of weight, loss of hair and deformations of the bones of the extremities were frequently observed during the last weeks.

After death in the animals with anaemia, the caecum and the beginning of the colon contained a brown-red coloured, semi-fluid mass. The latter consisted of remnants of foodstuffs and more or less destroyed blood. These high degrees of anaemia and intestinal haemorrhage developed in the first experiment in all four cases, in the second only once in an animal fed with cow's milk. One might believe that the wood shavings, by irritating the intestinal wall, promote the development of haemorrhage and in consequence the anaemia, but the case where no wood shavings were given proves that such irritation is by no means necessary.

Ulcers or other symptoms of inflammation of the intestinal wall were not found. Undoubtedly the blood originated from "capillary bleedings" as may be found in scurvy in a number of organs. Nevertheless the diagnosis of scurvy could not be supported by macroscopical examination of the deceased animals or by studying the microscopical appearance of the junctions of the bony and cartilaginous parts of the ribs. Indeed, some investigators [Nelson, Lamb and Heller, 1922] hold that rabbits need none or very little of the antiscorbutic factor; moreover, the food (raw milk) contained undoubtedly a certain amount of vitamin C[1].

It cannot be stated positively that the intestinal haemorrhages were the only cause of the anaemia. It was often observed that the faeces of the living animals were darkly coloured; but the blood-reaction was very faint (benzidine reaction), in distinction to what was found in the caecum-content after death.

Summary.

Not only in human milk-anaemia, but also in experimental milk-anaemia (by cow's or goat's milk), one should not only make allowance for *decreased* building up of haemoglobin. For a symptom (*urobilinuria*) may be found that indicates (but not yet proves) an *increased* loss of red blood corpuscles; and in the deceased experimental animals (rabbits) an *increased* loss of red blood by capillary haemorrhage into the intestinal cavity was observed.

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[1] In winter, by giving sugar beets to the milk-giving animals, the presence of vitamin C in their fodder was secured.

XIX. A CHEMICAL STUDY OF LEAF CELL CYTOPLASM.

I. THE SOLUBLE PROTEINS.

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(*Received December 31st, 1925.*)

Our knowledge of the chemistry of vegetable cytoplasm rests primarily on the classic research of Reinke [1881] on the cytoplasm of the slime-mould *Aethaleum septicum*. This simple organism, which has no cell wall and contains a very large number of nuclei, is highly specialised, and it is doubtful if plant physiologists have any right to assume, as they have done, that the cytoplasm of the normal vacuolated plant cell has a similar composition.

The present research has been undertaken with the object of gaining some insight into the composition and physical properties of leaf cytoplasm. Such knowledge should not only throw light on the metabolism of the cell but should also help to explain the mechanism of cell permeability. The cytoplasm from vacuolated cells which do not contain those highly specialised inclusions—the chloroplasts—would have been preferable in some respects, but leaves have been chosen, not only because they can be obtained in large quantities, but also because the elasticity of the cell wall enables the cytoplasm to be extracted in a state free from cell wall and vacuole material.

In the investigations reported in the present paper it has been necessary to treat the leaves as a whole unit. The objections to this are obvious: not only may the individual cells vary in composition, but the chloroplasts and other inclusions are not differentiated from the protoplasm. At the present stage of the investigation this cannot be avoided, for, from the practical standpoint, it is impossible to obtain a good preparation of cytoplasm *in bulk* from unicellular organisms. If we are successful in obtaining a fairly comprehensive analysis by the methods now employed, it may be possible at a later stage of the investigation to apply micromethods to individual cells.

Before making as complete an analysis as possible of the cytoplasm from one particular species of leaf, we have thought it advisable to make a general survey of the relations between the cytoplasm and sap of a large number of different species. The ease with which the cytoplasm can be extracted from the leaf cells, the amount of "combined" and "soluble" protein that the cytoplasm contains and the relation of the isoelectric point of the proteins

to the hydrogen ion concentration of the leaf sap form the basis of the present communication. More comprehensive analyses of the proteins, substances soluble in alcohol (fats, lipins, etc.) and the inorganic material of leaf cytoplasm are in progress.

General Experimental Methods.

Materials employed. The leaves were gathered on the morning of the experiment and used within one or two hours.

Methods of manipulation (separation of cytoplasmic proteins and vacuole material). *Method A.* Details of this method have already been published [Chibnall, 1924, 1] and need only brief reference here. The leaves were first cytolysed with ether, enveloped in filter-cloth and pressed in the Buchner press. The leaf residues were then washed free from the remainder of the vacuole material by alternate soaking in water and pressing. The cytoplasm, from which some water-soluble substances may have been removed, was then dispersed into colloidal solution by grinding with water in a meat chopper, the cellular matter being afterwards strained off by squeezing through fine gauze. This colloidal solution of cytoplasm contains protein, which it is possible to separate into two fractions by filtration through paper pulp. That fraction retained on the filter pad is in some loose combination with substances soluble in alcohol, whilst that in the filtrate appears to be uncombined and present in solution as an anion. This would seem to be shown by the fact that it can be precipitated by the addition of acid. It is probable that both these fractions contain more than one protein and, until further research has enabled a satisfactory classification of the leaf proteins to be drawn up, it is proposed to refer to these fractions simply as the "combined" and "soluble" proteins respectively. The present paper deals almost exclusively with the "soluble" proteins extracted from different leaves.

Some leaves—from which a large amount of cytoplasm, mixed with vacuole material, could be extracted by simple grinding—gave only a small amount of colloidal cytoplasm when ground up after the ether treatment already described. What appeared to be some form of denaturing of the cytoplasmic proteins after cytolysis of the cells with ether had already been observed when investigating the leaves of *Medicago sativa* [Chibnall, 1924, 2]. To gain some insight into the amount of this supposed denaturing, a certain number of the different species of leaves were treated in the following way.

Method B. About 200 g. of fresh leaves were minced in a "Universal No. 2" mincing machine, the material being returned to the machine for a second grinding. The minced pulp was then wrapped in a small piece of stout filter-cloth and the liquid expressed in the Buchner press. The press residue was then allowed to soak up water and the operation was four times repeated. The combined extracts were made up to 500 cc. and total-N determined on an aliquot part. The remainder of the solution was filtered through a small, but thick, pad of paper pulp, which was washed with water

until the total volume of the filtrate was 1000 cc. Total-N was then determined. The difference between these two nitrogen values, after allowance for the liquid withdrawn for aliquots, gives the N of the "combined" protein retained on the filter pad. The remainder of this solution was boiled to coagulate protein and again filtered. The filtrate and washings were made up to 1000 cc. and total-N determined. Allowing for aliquots withdrawn the total-N in this last solution represents the water-soluble non-protein-N, and the difference between this value and the one immediately preceding represents the N of the coagulated ("soluble") protein. Examples of such analyses are given later in Table V.

In connection with the above procedure two points must be noted. The coagulated protein from the first filtered extract will contain not only the "soluble" protein of the cytoplasm, but also a small amount of protein that appears to be present normally in the vacuole fluid. Whilst the former seems to represent between 15 % and 25 % of the total leaf-N it has been our experience that the latter represents only about 2 %; therefore in the present paper the coagulated-N can be taken as representative of the "soluble" proteins of the cytoplasm. Secondly the success of this separation depends entirely on the efficiency of the filter pad. Very fine pulp, rammed down to a depth of one to one and a quarter inches on a six inch Buchner funnel, has been used. Filtration through such a pad is necessarily slow, but experience has shown that a more loosely packed pad allows a considerable amount of the "combined" protein to pass through. The filtered leaf extracts should be brown, without any green tinge; whilst the coagulum of protein should be grey or white. If the coagulum is ground up with 10 cc. of 90 % alcohol and filtered, the filtrate should be either colourless or only faintly green. If more than a trace of chlorophyll is present, then some of the "combined" protein has passed through the original filter pad.

Hydrogen ion concentration of the leaf sap.

Hydrogen ion concentration was determined in all cases electrometrically, using a Cambridge Instrument Company's standard portable potentiometer box and a Hildebrand type of hydrogen electrode. Readings were frequently checked with a glass electrode [Kerridge, 1925], which is not subject to the errors due to the bubbling of hydrogen through the liquid, or the "killing" of the platinum electrode which occasionally occurs when working with solutions containing protein.

When possible the hydrogen ion concentration of the sap prepared by both the methods outlined above has been determined. It is more convenient to discuss the sap given by Method B first. When the leaf cells are ruptured by grinding and the resulting pulp pressed out, a thick green liquid is obtained, containing (1) the contents of the vacuole and (2) part, but not all, of the cytoplasm, which has become colloidally dispersed in the vacuole liquid. The hydrogen ion concentration of this sap then does not represent

that of either the cytoplasm or the vacuole fluid in the living cell, but a mixture of both these constituents extracted from the dead cell.

Previous experimental work has shown that the extract obtained by pressing the cytolysed leaves (Method A) consists of the vacuole fluid, mixed, possibly, with some of the more easily diffusible constituents of the cytoplasm. It might be thought that the presence of ether would affect the hydrogen ion concentration as determined by the hydrogen electrode, but experience has shown that any change produced is not within the limits of accuracy required for the present work. For example, *Heracleum sphondylium* leaf sap prepared by Method B was at p_H 6·19. After shaking this sap with 1/15th its volume of ether the hydrogen ion concentration was unchanged.

In the case of all the leaves investigated the hydrogen ion concentration of the cell contents given by Method A and the vacuole material given by Method B do not differ by more than p_H 0·2; generally the agreement is very much closer. This result shows that even if the cytoplasm and vacuole fluid in the *living* cell are at different hydrogen ion concentrations, the permeability of the cytoplasm after cytolysis rapidly brings about equilibrium between them. Therefore the hydrogen ion concentration of the sap given by both methods depends on the relative buffering power of the cytoplasm and the vacuole fluid. Our experience leads us to conclude that the buffering power of the latter is relatively much greater than that of the former so that the observed hydrogen ion concentration is a close approximation to that which would be given by the vacuole fluid in the living cell. In a later paper the relation between the hydrogen ion concentration of the cytoplasm and vacuole fluid will be discussed in much greater detail; it may be mentioned that there is already evidence that a gradient of hydrogen ion concentration exists across the cytoplasmic membrane.

Table I shows the hydrogen ion concentration of the cell sap from a number of different leaves (Method B). The variations between different samples of the same species of leaf are surprisingly small, and seldom exceed p_H 0·2. In cases where the leaves were partially or wholly etiolated greater variations were found. Thus green leaves from unheaded cabbage gave p_H 5·6 to 5·7, whereas leaves from headed cabbages varied between p_H 6·0 and 6·4. As no attempt has been made to inquire into seasonal variations, the relative ages of the plants when the leaves were picked has not been quoted.

Preparation of the "soluble" proteins.

It has not been thought necessary to give details of every preparation made; a condensed summary of one preparation from each species of leaf is given in Table II. The details for *Spinacea oleracea*, *Medicago sativa* and *Zea Mays*, which have already been published, are included for purposes of comparison.

The percentage of N shows that the purity of the protein extracted by the same method from different leaves varies. The first six proteins enumerated

Table I. *The hydrogen ion concentration of the leaf sap.*

(Figures marked * are for sap prepared by Method A; remainder by Method B.)

Species	p_H of sap
Ficus Carica (fig)	6·87 6·85* 6·73
Spinacea oleracea (spinach)	6·57 6·57* 6·57* 6·53* 6·50
Helianthus multiflorus	6·54
„ *annuus* (sunflower)	6·40
Heracleum sphondylium (cow-parsnip)	6·19 6·30*
Tetragonia expansa (New Zealand spinach)	6·06 6·08*
Phaseolus multiflorus Willd. (scarlet runner)	5·97 5·92* 6·12 6·09 6·04 6·00
Populus tremula	5·93
Atropa Belladonna (deadly nightshade)	5·80
Clerodendron trichotomum	5·83 5·78 5·62 5·72* 5·47
Wistaria chinensis	5·70
Vicia Faba (broad bean)	5·67 5·84*
Medicago sativa (lucerne, alfalfa)	5·67
Zea Mays (maize)	5·66
Brassica oleracea (cabbage) "unheaded"	5·60 5·60* 5·68
Tropaeolum majus (nasturtium)	5·60
Crambe cordifolia	5·56 5·50*
Catalpa bignonioides	5·50 5·57*
Cochlearia Amoracia (horse-radish)	5·37 5·40 5·41 4·98* 5·20* 5·45 5·05*
Acer pseudoplatanus (sycamore)	4·88 4·64*
Polygonum cuspidatum	4·52
Saxifraga cordifolia	4·22
Rheum rhaponticum (rhubarb)	4·00 4·62* 4·36*
Pterocarya caucasia	4·15
Parthenocissus quinquefolia	3·70
„ *tricuspidata*	3·56
Rumex acetosella	3·19
Vitis vinifera (vine)	3·21 3·04 3·02* 3·03* 3·12

in the table do not give the Molisch test for carbohydrate; the remainder give a small amount of furfural on boiling with hydrochloric acid, and a carbohydrate impurity of the nature of pectin is possibly present. This impurity does not seem to denature in the same way as the cytoplasmic proteins, because the smaller the yield the less pure is the "soluble" protein from the same species of leaf. In some cases, where the yield of protein by Method A is small, as in *Cochlearia Amoracia*, the protein prepared by Method B has a higher N content (N 15·70 %, ash-free). As a general rule it has been found that the amount of impurity increases as the leaf ages.

The leaves of *Helianthus multiflorus*, *Tropaeolum majus* and *Atropa Belladonna* have been examined qualitatively. The cytoplasm was easily extracted by Method A and soluble proteins were prepared from them.

Analysis of the "soluble" proteins.

Table III gives the analyses of the proteins by Van Slyke's method, as recently modified by Plimmer and Rosedale [1925]. The analyses show that a close relationship exists between all these proteins, the only significant variations being in the amide-N and histidine-N.

Theoretically it should be possible for a protein to undergo amidisation or deamidisation without any change in the internal structure of the protein molecule. Such little evidence as has yet been collected shows that these proteins undergo deamidisation as the leaf becomes more mature. Thus the

amide-N of a preparation from young leaves of *Phaseolus multiflorus* was 7·36 %, from mature leaves 5·69 %, from young leaves of *Heracleum sphondylium* 7·95 % and from aged leaves 6·46 %. In the latter case no significant change was observed in the distribution of the basic-N.

Table II. *Showing details of "soluble" proteins prepared by Method A.*

Species	Weight of leaves taken kg.	Yield of "soluble" protein g.	"Soluble" protein Ash %	"Soluble" protein Ash-free N %
Spinacea oleracea	22·7	70·0	1·72	16·25
Vicia Faba	3·0	6·1	4·27	16·77
Phaseolus multiflorus	2·2	16·0	3·12	15·85
Heracleum sphondylium	4·3	11·8	6·20	15·90
Medicago sativa	5·5	32·3	0·20	15·76
Crambe cordifolia	3·0	7·0	2·20	15·70
Brassica oleracea	1·5	2·0	5·90	14·65
Zea Mays	6·7	29·6	3·28	14·40
Cochlearia Amoracia	2·5	1·0	1·28	14·37
Tetragonia expansa	5·5	5·0	5·07	14·46
**Helianthus annuus* (Method B)	2·4	14·2	10·4	13·70
Ficus Carica	3·0	4·0	9·7	13·40

* Old leaves showing signs of chlorophyll degeneration.

Table III. *Van Slyke analysis of the "soluble" proteins.*

(In percentages of total nitrogen; nitrogen figures corrected for solubility of bases.)

	Spinacea oleracea	*Medicago sativa*	*Zea Mays*	*Vicia Faba*	*Cochlearia Amoracia*	*Crambe cordifolia*	*Brassica oleracea*	*Tetragonia expansa*	*Ficus Carica*	*Heracleum sphondylium*	*Phaseolus multiflorus*	*Helianthus annuus* (aged leaves)
Amide-N	6·93	5·51	7·44	7·49	6·37	6·51	6·37	7·36	6·96	7·95	5·69	6·82
Acid humin-N	0·76	1·22	1·91	1·87	2·36	2·16	1·74	1·81	3·99	2·40	3·12	3·61
Basic humin-N	1·71	1·48	2·66	1·51	1·99	1·34	1·29	1·93	2·99	1·90	1·06	4·92
Arginine-N	13·80	15·32	14·69	16·08	14·44	16·75	14·29	15·75	14·54	15·15	16·08	13·08
Histidine-N	3·89	3·09	4·70	9·02	5·23	7·30	7·05	8·15	7·36	10·20	6·24	6·45
Lysine-N	10·47	10·28	9·07	6·99	8·82	8·84	10·07	8·15	8·84	7·60	10·07	6·77
Mono-amino-N	58·42	59·11	56·29	50·00	55·24	53·07	56·87	53·93	50·68	50·36	53·85	52·46
Non-amino-N	2·58	3·19	2·04	6·90	4·23	3·20	1·51	2·39	1·50	4·00	3·40	3·23
	98·66	99·20	98·80	99·86	98·68	99·17	99·19	99·47	96·86	99·56	99·51	97·34
Total basic-N	28·16	28·69	28·46	32·09	28·49	32·89	31·41	32·05	30·74	32·95	32·39	26·30

This variation of amide-N, which has not been observed in any of the seed proteins, may be due to the fact that the "soluble" proteins are mixtures. In spite of this, however, the general agreement between the analyses given in Table III shows that the composition of this fraction of the cytoplasm is very similar in all the leaves investigated.

Properties of the "soluble" proteins.

The proteins extracted from the different leaves outlined above are similar in most respects to that from the leaves of *Spinacea oleracea*. They all belong to the ill-defined class known as glutelins.

Between the limits p_H 4·0 and 5·0 the solubility of the proteins is at a minimum and in the case of spinach is of the order 0·0014 %. Below p_H 4·0

the proteins are slowly soluble, the solubility increasing with decrease of p_H to 2·5, beyond which they are again precipitated. In such an acid solution the proteins are sensitive to the presence of salts, though not all to the same degree. The protein from spinach will dissolve with difficulty at about p_H 3·0 in the vacuole sap from the same leaves, whereas those from cabbage and horse-radish will not dissolve in an acid solution containing as little as $M/50$ sodium chloride.

Above p_H 5·0 the proteins are readily and increasingly soluble with increase of p_H, and are precipitated from solution only by salts which are used for this purpose in general protein chemistry (ammonium sulphate, etc.).

The isoelectric point of the "soluble" proteins.

The isoelectric point has been determined by the method of maximum precipitation. The original protein solution as prepared by Method A has a p_H between 6 and 7. On account of the rapid denaturing that some of these proteins undergo in acid or alkaline solution the isoelectric point was determined with as little delay as possible. 50 cc. portions were run into a series of graduated tubes and increasing amounts of $N/10$ hydrochloric acid added to them successively. In all the preparations made the protein was completely precipitated between the limits p_H 4·0 and 5·0 within one quarter of an hour; on standing a few hours some of them were completely precipitated over a range as wide as p_H 3·5 to 6·5. Attempts to obtain a narrower range for the isoelectric point by using buffered solutions and measuring the volume of precipitated protein led to inconclusive results. It is possible that the wide precipitation zone of these proteins, like that of the globulin edestin, is due to the relative insolubility of the protein acid compounds in the neighbourhood of the isoelectric point [cf. Cohn, 1925]. Alternatively, as there is no evidence that the proteins being investigated are not mixtures, the range p_H 4·0 to 5·0 may represent the limits within which the constituent proteins of the mixture are isoelectric.

The p_H of the sap from which these proteins were extracted varied from 6·87 in the case of *Ficus Carica* to 5·37 in the case of *Cochlearia Amoracia*. Therefore the proteins must have been present in the cytolysed cells as anions, but at a p_H not far removed from that of their isoelectric point. The ease with which the cytoplasm dispersed into colloidal solution immediately after the cells were broken open indicates that the proteins in the living cell were present also as anions.

The denaturing of the "soluble" proteins.

The (presumed) denaturing which these proteins undergo in certain circumstances has been the cause of considerable trouble during the present investigations. By Method B, which separates the "soluble" protein from the cellular material and the remainder of the cytoplasm within a few minutes,

the "soluble" protein represents between 15 % and 25 % of the total leaf-N. But by Method A, which requires an hour or more for the ether cytolysis, pressing and washing, the yield is often very low, due to some change in the character of the cytoplasm, rendering it less easy to disperse into colloidal solution. A comparison of the yields of "soluble" protein by the two methods is given in Table IV.

No satisfactory explanation of this denaturing has yet been found. It is not caused by the ether used in the cytolysis, for the expressed cytoplasm obtained by Method B, if shaken with ether to the point of saturation, has no greater tendency to flocculate than a similar extract not so treated. Although the hydrogen ion concentration of the vacuole fluid may have some effect, it is not the determining factor, for leaves of the runner bean, the proteins of which do not appear to undergo any denaturing, have a vacuole fluid at p_H 6·0; whilst the leaves of New Zealand spinach, the proteins of which denature very rapidly, have a vacuole fluid at p_H 6·08. Further, it has been found by experience that the pressure applied in the Buchner press has no appreciable effect on the yield of "soluble" protein. Thus no factor which will account for this denaturing has been found, and it is possible that the denaturing is due to differences between the individual leaf cells, the vacuole fluids of which diffuse from one to another after cytolysis.

Table IV. *Comparison of the yields of "soluble" protein.*

(Protein-N as a percentage of total leaf-N.)

Species	Method A %	Method B %
Spinacea oleracea	14·1	16·7
Vicia Faba	4·0	—
Phaseolus multiflorus	16·0	16·4
Heracleum sphondylium	5·1	—
Medicago sativa	8·6	15·1
Crambe cordifolia	6·0	27·0
Brassica oleracea	4·0	15·4
Zea Mays	12·0	—
Cochlearia Amoracia	1·0	19·4
Tetragonia expansa	4·5	17·5
Helianthus annuus	—	13·7
Ficus Carica	1·5	—

The rôle of the "soluble" proteins in the metabolism of the leaf cell.

As the amount of "soluble" protein extracted from the leaf must necessarily depend on the number of ruptured cells, the yields given in Table IV are minimum values. When the methods now being employed for the extraction of the cytoplasm were being investigated, the various operations were frequently followed with the aid of the microscope. There is no doubt that the N remaining in the leaf residues is due to unextracted cytoplasm, which has not been scoured out of the ruptured cells by the grinding. Within the limits of accuracy possible at this stage of the investigations there seems no reason to assume that this unextracted cytoplasm differs in composition

from that which has been extracted. Table V shows how the yield of "soluble" protein compares with that of the "combined" protein. It might at first be thought that, as the "soluble" protein is not in any apparent combination with the fats, etc. in the cytoplasm, it is not necessary for the vital functions of the living cell and that its sole function is to act as a reserve of N which can be utilised by the cell as required. The following experiment does not support such a contention, and shows that the functions of the "soluble" and "combined" proteins in the leaf are not dissimilar.

Some leaves of *Phaseolus multiflorus* with long petioles were cut from the plant and left exposed to diffuse daylight for four days with the petioles dipping in water. Previous experiment [Chibnall, 1924, 3] had shown that under these conditions considerable degradation of the cytoplasmic proteins occurs, giving rise to soluble products, chiefly asparagine. Table VI shows the distribution of the protein-N and non-protein-N obtained by Method B. The non-protein-N has increased from 15·8 % to 28·5 %, yet the ratio of "soluble" to "combined" protein extracted shows no great variation. As mentioned above, there is no reason to assume that the cytoplasm unextracted by the methods employed differs materially from that which has been extracted, and it would appear therefore that the increase of non-protein-N has taken place at the expense of both the protein fractions of the cytoplasm, and not from the "soluble" protein alone.

Table V. *Showing the amounts of "combined" and "soluble" protein extracted from various leaves.*

(Percentage of total leaf-N.)

Species	*Cochlearia Amoracia*	*Phaseolus multiflorus*	*Brassica oleracea*	*Spinacea oleracea*
"Combined" protein-N	18·4	35·4	14·0	19·1
"Soluble" protein-N	19·4	16·4	15·4	16·7
Water-soluble non-protein-N	13·5	15·8	31·7	26·7
Unextracted-N	48·7	32·4	38·9	37·5

Table VI. *Showing the change in the distribution of N in leaves placed with their petioles in water.*

Phaseolus multiflorus.

(Percentages of total leaf-N.)

	Leaves kept with their petioles in water for 4 days	Freshly picked leaves
"Combined" protein-N	33·5	35·4
"Soluble" protein-N	14·9	16·4
Water-soluble non-protein-N	28·5	15·8
Unextracted-N	23·3	32·4

Leaves from which no "soluble" protein could be prepared.

No "soluble" proteins were extracted from the following two groups of leaves by the methods outlined above.

Group 1. Leaves of *Populus tremula*, *Wistaria chinensis*, *Rheum rhaponticum*, *Clerodendron trichotomum*, *Polygonum cuspidatum*, *Saxifraga cordifolia*, *Catalpa bignonioides* and *Pterocarya caucasia*. By Method B an extract charged with cytoplasm was obtained, which flocculated fairly readily on standing. The filtered extract gave but very little coagulum on boiling. Method A gave only a very small amount of cytoplasm. In the case of leaves with a fairly acid sap, such as rhubarb, p_H 4·0, a considerable amount of "soluble" protein could be obtained by using $N/100$ sodium hydroxide in place of distilled water during the grinding which extracts the cytoplasm. In the case of rhubarb the yield corresponded to 6 % of the total leaf-N, the preparation containing 5·7 % of ash and, ash-free, 14·3 % of N. This protein flocculates fairly sharply on adding acid at about p_H 3·5, and is insoluble in excess of acid. It is probable that part, at least, of this preparation is derived from the "combined" protein by the mild hydrolytic action of the alkali, for the alcoholic washing contained solids equivalent to 11 % of the final preparation.

Group 2. Leaves of *Parthenocissus quinquefolia*, *Parthenocissus tricuspidata*, *Rumex acetosella* and *Vitis vinifera*. These leaves are distinguished from all the leaves mentioned above, not only by the extreme acidity of the sap, but also by the fact that they contain a considerable amount of what appears to be a mucilage. The presence of this material interferes with the extraction of the cytoplasm both by methods A and B: some modification of the technique employed will have to be devised before the cytoplasmic proteins can be extracted in a state of purity.

Reviewing all the above results it will be seen that we have been able to extract proteins in a state of purity only from the leaves of herbaceous plants having a leaf cell sap which is alkaline with respect to the isoelectric point of the proteins.

Summary.

The proteins of leaf cytoplasm can be separated into two fractions: (A) "combined" proteins, which are in some loose combination with substances soluble in alcohol, and (B) "soluble" proteins, which are uncombined, and which pass fairly readily into solution when the cytoplasmic gel is ground with water.

The "soluble" proteins of a large number of different leaves have been investigated. Preparations sufficiently pure for chemical analysis have been made from the leaves of eleven herbaceous plants and one ligneous plant.

These proteins are glutelins, and although they may be mixtures, their chemical properties are very similar. All of them have an isoelectric range from p_H 4·0 to 5·0 in which their solubility is at a minimum. The hydrogen ion concentration of the leaf cell sap was in all cases alkaline with respect to this isoelectric range, so that the proteins were present in the cytolysed cells, and probably in the living cells, as anions.

Brief mention is made of leaves from which it has not yet been possible to prepare pure "soluble" proteins.

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XX. SOURCES OF ERROR IN THE TECHNIQUE EMPLOYED FOR THE BIOLOGICAL ASSAY OF FAT-SOLUBLE VITAMINS.

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The dual character of the fat-soluble vitamins, which was discovered by McCollum and his co-workers [1922], has thrown doubt upon the validity of much previous work involving their titration. This discovery has been confirmed by many subsequent workers and it has now been accepted generally that fat-soluble A, the vitamin concerned with prevention of ophthalmia and respiratory disorders in rats, must be studied separately from the anti-rachitic substance, or vitamin D, which occurs in association with vitamin A in many naturally occurring fats and oils.

Steenbock and Nelson [1923] were the first to point out that to designate vitamin A as the "growth factor" in any special sense was inappropriate. They brought forward evidence which they have since greatly amplified to show that the anti-rachitic vitamin is also necessary for the maintenance of growth in young animals. In short, the view of these workers is that both vitamins A and D are to be regarded merely as two members of a whole group of essential factors in diet, any one of which may limit growth, notwithstanding the presence of all the others.

This constitutes a serious criticism of much of the previous work upon fat-soluble A, notably that concerned with the evaluation of this vitamin in various foodstuffs. It is probable that much of our present knowledge of the distribution of vitamin A in nature will need revision. Hitherto the criterion has been the growth induced after administration of the substance in question to young rats upon a diet deprived of *both* fat-soluble vitamins, as, for example, in the method of Zilva and Miura [1921].

Steenbock and his co-workers maintain that vitamin A can only be estimated appropriately under circumstances in which the experimental animal is provided with an adequate supply of the anti-rachitic substance, as, for example, by irradiation with ultra-violet light. They have, therefore, recommended a new method for determination of vitamin A [Steenbock, Nelson and Black, 1924]. The young rats, placed upon a diet deprived of fat-soluble vitamins, are at the same time irradiated daily with the rays from a mercury vapour quartz lamp. The degree to which growth is maintained after addition of various foodstuffs is then a measure of the vitamin A content of these[1].

[1] Note added Feb. 8th. Drummond, Coward and Handy [1925] have recently described a similar method in which the anti-rachitic vitamin is supplied to the diet in the form of irradiated crystals of pure cholesterol.

Not only do these new facts and theories require a re-examination of much previous work in this field, but they also serve to explain much previous confusion.

There was a whole series of facts which made the dual character of the fat-soluble vitamins less easy of acceptance. Their parallel distribution in nature and the apparent possibility of replacing vitamin A as well as vitamin D by ultra-violet light [Hume, 1922; Goldblatt and Soames, 1922] seemed to argue, if not unity, at least some close relationship. Cod-liver oil, for example, the richest known source of vitamin A, was found to be also the richest source of vitamin D. Goldblatt and Zilva [1923], while investigating the destruction by heat and oxidation of the two fat-soluble vitamins in cod-liver oil, attempted to titrate both factors separately in the original oil. Using the method of Zilva and Miura [1921] they found the minimum daily dose required to restore growth in rats upon a diet devoid of fat-soluble vitamins to be 2·2 mg., and that needed to protect similar rats from rickets while receiving McCollum's No. 3143 rickets-producing low-phosphorus diet, to be 3·3 mg. The discrepancy between these two figures is not great when account is taken of the large experimental error inseparable from work of this kind. It is, however, possible in the light of the theories advanced by Steenbock and his colleagues that, in this estimate of vitamin A, the restored growth of the rats may have been limited by the animals' supply of the anti-rachitic vitamin D, *i.e.* that received together with the vitamin A in the dose of cod-liver oil, as well as what could be mobilised from any reserves that the animal may have possessed. If during the test the experimental animals had been provided with excess of anti-rachitic substance from another source, by means of irradiation or otherwise, it is possible that growth might have been restored with a smaller dose of oil. If, on the other hand, the experimental animals when first placed upon the deficient diet possessed reserve stores of vitamin A in excess of those of vitamin D (which would appear to be a usual condition), the failure to grow after 2–3 weeks might have been due to exhaustion of vitamin D reserves. The size of the cod-liver oil ration required to restore growth would then be a measure not of the vitamin A, but of the vitamin D contained in the oil. The result (minimal effective dose 2·2 mg.) published by Goldblatt and Zilva then shows a satisfactory concordance with that obtained by them using the more direct method of measuring vitamin D, viz. 3·3 mg., the dose required to prevent occurrence of rickets in young rats upon a standard rickets-producing diet, and the coincidence that vitamins A and D are present in equal concentration disappears. In any case the results cannot now be regarded as proof that cod-liver oil contains both vitamins in approximately equal concentration.

These new theories regarding the interrelation of the two fat-soluble vitamins have also helped to explain difficulties and irregularities which were suspected to lie in errors of technique but which were difficult to trace. One such irregularity forms the subject of the present paper.

In this Institute, in the course of work extending over several years, we have found a great deal of unexplained irregularity in the behaviour of our stock rats when placed upon diets deprived of fat-soluble vitamins. Although all the animals are bred in the Institute under apparently constant, standardised conditions of management and diet, a large variation has been noticed in the time elapsing after the young rats are placed upon the experimental diet before they cease to grow. This is shown in Table V, where are collected data, obtained from winter 1923–4 to the autumn of 1925, concerning the growth in weight, and calcification of bones, of young rats placed on diets devoid of fat-soluble vitamins at 25–35 days old and of weight 40–55 g. In the 6th column of Table V are given the average weights after 21 days upon the experimental diet for male and female rats, respectively, of the different litters. In the 7th column are given the maximum weights attained. Only litters in which 6 rats and over survived are included in the table. Fig. 1 (p. 128) shows graphically the variation in percentage increase in weight after 21 days upon the experimental diet.

In the course of attempts to discover the cause of the irregularity, a detailed investigation has been made of every stage in our technique for estimating fat-soluble vitamins and the results are set out in the following pages. In general, the irregularity appears to have some connection with the time of year in which the experiments are carried out, the young rats on the deficient diet showing a greater impulse toward growth, and better calcification of their bones, in summer and autumn than in winter and spring. The animals under experiment are kept in rooms with glass windows facing north, and except in early morning in summer, no direct sunshine reaches the interior. Still, the amount of illumination varies greatly at different times of the year and it was thought that this might be significant. However, the results of special observations made to investigate this point showed that it was unimportant.

Attention was next turned to the experimental diet, to see whether there might not be irregularities introduced in the details of the constituents or of their preparation. The results here were also negative.

Finally, the possibility was explored that there might be unrecognised variations in the breeding stock and in the state of nutrition of the young animals themselves at different periods of the year. It is in this connection that we have found the theories put forward by Steenbock and his co-workers to be helpful in guiding us to what we now believe to be the real cause, viz. variations in the reserves of the two fat-soluble accessory factors, and in particular of the anti-rachitic vitamin, with which the young are equipped at the beginning of their independent existence. Numerous observations have led to this conclusion and are set out below in detail. They are collected from the experiments of various workers in this Institute. Special thanks are due to Miss Hume, Prof. Korenchevsky and Dr Zilva for placing the results of their work at my disposal for publication in this paper.

Influence of the degree of illumination of the animals under experiment.

A litter of 12 young rats, born in the month of June, was divided directly after weaning into two groups of 6 rats each. In each group 2 of the 6 animals received cod-liver oil and acted as controls. All received a diet deprived of fat-soluble vitamins, but the two groups were subjected to different degrees of illumination. Both groups of rats were indoors behind glass windows, the better illuminated animals occupying a well lighted laboratory with a north aspect, whilst the poorly illuminated ones were placed in a darkened corner screened from all direct sunlight. The experiment was made in bright weather, during July and August 1924, and lasted 7 weeks. Rough comparative measurements have been made of the illumination of the two situations by means of a simple photometer (Eder-Hecht Dauer-Graukeil-photometer [see Hecht, 1918]), and it was found that the illumination of the darker position was not more than about 1/200th of that of the brighter one.

The results showed little difference between the animals of the two groups either in the degree of growth achieved or the degree of calcification of the bones. The calcium content of the dissected leg bones was determined by the method of Aron and Sebauer [1910] and the amount calculated on the dry weight of the bone. Phosphorus was determined in the pooled blood of the animals in each group, using Briggs' [1922] modification of the method of Bell and Doisy with the precautions described by Martland and Robison [1924]. The differences between animals of the two groups were insignificant and within the error of experiment. The advantage, if any, was in favour of the better illuminated animals (see Table I).

In a second experiment (see Table II), also carried out during July and August 1924, the diet was modified to contain white wheat flour in place of wheaten starch. Here again there was no significant difference between the animals in the two groups after a period of 50 days upon the experimental diet.

Investigations of the basal diet.

Attention was next directed to the possibility of variation in the constituents of our basal diet, more especially as regards the fat and caseinogen employed. The diet usually employed in this Institute, known as "F" diet, contained

Inactivated caseinogen	100 g.
Wheat starch	250 g.
Marmite	25 g.
Hardened cotton seed oil (or sesame oil) ...	75 g.
Lemon juice	25 cc.
Salt mixture	25 g.
Water	250 cc.

In some cases egg-white was used to replace 90 % of the caseinogen. This was tried in order to minimise error, when it was feared that the heating

Table I. *Influence of degree of illumination.*

Litter of 12 young rats of weight 25–31 g., aged 21 days. Maintained on diet F-R deprived of fat-soluble vitamins and containing 0·27 % Ca and 0·37 % P, reckoned on dry weight of the diet.

Duration of experiment = 52 days. July–August, 1924.

(*a*) **In well-lighted laboratory.**

		Body weight				Analysis of bones		Blood P* mg. per 100 cc.				
Rat No.	Sex	Initial g.	Max. or final g.	Increase %	Mean	Time days	H_2O %	Ca on dry wt. %	Inorg.	Total acid-soluble	Ribs No. of fractures	Histology of rib-junctions
65	♀	30	71	137 134	135	52	54	15·5	—	—	Infractions present	Osteoporosis
66	♀	25	59	—		52	53	16·6	6·6	38·8	0	Osteoporosis
67	♂♀	30	86	187	160	52	51	16·0			0	Osteoporosis and very slight rickets
68	♂	29	68	134		52	50	15·0			0	Osteoporosis and ? very slight rickets
						Mean	**52**	**15·8**				
69	♂	31	154	397	381	52	47	19·7	6·4	46·3		Practically normal
70	♂	26	121	365		52	46	19·7				Practically normal
						Mean	**46·5**	**19·7**				

cod-liver oil added to the diet

(*b*) **In dark corner, screened from direct daylight.**

71	♀	31	85	174		52	52	17·0			0	Osteoporosis
72	♀	28	60	—		34	—	—				
73	♂♀	31	93	200	153	52	53	17·2	6·3	38·1	2	Osteoporosis and slight rickets
74	♂	30	62	107		52	55	14·8			8	Osteoporosis and slight rickets
						Mean	**53**	**16·3**				
75	♂	28	127	353	345	52	59	18·8	6·4	43·4		Nearly normal but more osteoporosis in comparison with 69 and 70
76	♂	29	127	338		52	46	15·8				
						Mean	**47**	**17·3**				

Cod-liver oil added to the diet

NOTE. No. 72 found dead and partly eaten on the 34th day; thereafter growth of 73 and 74 much improved

* Animals killed fasting, fed only on previous day; analyses made on the pooled blood of the group.

of the caseinogen might not have been effective in destroying all traces of fat-soluble vitamins.

Table II. *Influence of Degree of Illumination.*

Litter of 10 young rats of weight 27–33 g., aged 21 days. Maintained on diet deficient in fat-soluble vitamins (F diet with starch replaced by white flour).
Duration of experiment = 50 days. July–August, 1924.

Rat No.	Sex	Body weight: Initial g.	Body weight: Max. or final g.	Body weight: Increase %	Mean	Analysis of bones: Ca on dry wt. %	Blood P* mg. per 100 cc.: Inorganic	Blood P* mg. per 100 cc.: Total acid soluble
(*a*) In well-lighted laboratory.								
77	♀	27	96	255	256	19·19		
78	♀	30	97	223		19·24		
79	♀	29	115	296		19·33		
80	♀	27	95	252		18·60		
					Mean	**19·09**	**7·10**	**42·3**
81	♂	31	125	303	315	18·53		
82	♂	32	137	328		18·95		
					Mean	**18·74**		
					Mean value Group (*a*)	**19·0**		
(*b*) In dark corner, screened from direct daylight.								
83	♀	30	111	270	265	20·01		
84	♀	28	98	250		18·75		
85	♀	27	102	278		18·54		
86	♀	28	102	264		18·21	**6·80**	**43·9**
					Mean	**18·88**		
87	♂	33	123	282	291	17·15		
88	♂	29	116	300		17·78		
					Mean	**17·46**		
					Mean value Group (*b*)	**18·4**		

* Determined in the pooled blood of the whole group.

The salt mixture was No. 185 of McCollum and his co-workers [1917] and contained in 1000 parts:

NaCl	43·5	$CaH_4(PO_4)_2H_2O$	136
$MgSO_47H_2O$	137	Ca lactate ($5H_2O$)	327
$NaH_2PO_4H_2O$	87	Ferric citrate ($3H_2O$)	30
K_2HPO_4	240		

Fat. A large consignment of hardened cotton seed oil had served the needs of the Institute for some years, but when it was exhausted no more could be obtained and only hardened sesame oil was available. Special experiments therefore were made to determine whether the latter was equally devoid of fat-soluble vitamins and to compare it with the cotton seed oil used previously. The results given in Table III show that no consistent difference exists between the two oils judged either by the degree of growth or by the calcification of the bones. The mean calcium content in the leg bones (femur, tibia and fibula) reckoned on the dry weight was 14·5 % for animals receiving hardened cotton seed oil and 14·3 % for those receiving hardened sesame oil.

Table III. *The effect upon growth and calcification of bones of substituting hardened sesame oil for hardened cotton seed oil in the experimental diet.*

F diet in which 10 % of the protein is provided in the form of caseinogen and 90 % in the form of egg-white.

Duration of experiment = 40 days.

Litter	Rat No.	Sex	Body weight: Initial g.	Body weight: Max. or final g.	Body weight: Increase %	Duration of experiment days	Analysis of long bones (leg): H_2O %	Analysis of long bones (leg): Ca on dry wt. %	Character of fat employed
I	2	♂	50	89	78	40	50·3	13·0	Cotton seed oil
	3	♂	50	60	20	,,	45·8	11·8	,,
	7	♀	49	85	73	,,	51·7	15·1	,,
	1	♂	50	89	78	,,	47·4	14·2	Sesame oil
	4	♀	50	83	66	,,	48·0	14·5	,,
	5	♀	49	77	57	,,	48·4	13·2	,,
	6	♀	50	74	48	,,	45·2	13·8	,,
II	8	♂	57	91	60	38	48·6	14·3	Cotton seed oil
	9	♂	50	72	44	,,	48·4	14·7	Sesame oil
	11	♀	50	81	62	,,	45·6	16·3	,,
III	14	♂	51	103	102	38	47·4	16·3	Sesame oil
	12	♀	48	76	58	,,	48·0	15·0	,,
IV	19	♂	49	93	90	38	52·2	15·0	Cotton seed oil
	18	♀	50	73	46	,,	51·8	13·8	,,
	20	♀	49	82	67	,,	52·0	14·8	,,
	16	♂	50	90	80	,,	49·5	14·5	Sesame oil
	15	♂	50	93	86	,,	51·2	15·0	,,
	17	♀	49	58	18	,,	46·3	13·8	,,
			Normal for rats of the same age				44·0	20·5	

Caseinogen. The caseinogen used for experimental diets in this Institute is commercial caseinogen, prepared by precipitation with acetic acid. It is inactivated by being heated in an electric oven at 120–130° for 36 hours. The dry caseinogen is spread evenly on shallow trays and once every hour during the day-time is thoroughly raked over to expose fresh surfaces and to facilitate the oxidation of any traces of fat-soluble vitamins originally contained. It was conceivable that whilst vitamin A might be completely destroyed by such treatment, the anti-rachitic vitamin, which is known to be more resistant to heat and oxidation [McCollum, Simmonds, Becker and Shipley, 1922; Goldblatt and Zilva, 1923], might survive. Accordingly, diets made with caseinogen prepared in the above manner were compared with those containing caseinogen of the same batch, but heated at 120–130° for an additional period of 36 hours. During this total heating of 72 hours the caseinogen was raked over every hour during the three day-time intervals.

The results of three separate experiments made with three separate litters of young rats are given in Table IV. In each case one-half of the litter received the experimental diet prepared with the twice heated caseinogen. The time of observation varied from 8–12 weeks. In Exps. 1 and 3, comparing the males with males of the same litter and females with females, there does appear to be a slight inferiority in the bone calcification of the animals receiving the more drastically heated caseinogen. In Exp. 2, however, there

is no significant difference between the two groups in this respect. As regards growth in weight, the variation between the two groups may also be regarded as coming within the error of experiment. The blood phosphorus (inorganic and total acid-soluble) was determined in case of the animals of Exps. 2 and 3 and all the results were found to lie within normal variations. The values were slightly lower in the groups of animals receiving the more strongly heated caseinogen, but it is doubtful whether the differences are significant. A mild degree of xerophthalmia was apparent in most animals in both groups, but there was no apparent difference in the time of onset or severity of the symptoms.

Table IV. *Growth and calcification of bones in young rats on F diet deprived of fat-soluble vitamins. Influence of degree of heating undergone by the caseinogen incorporated in the diet.*

			Body weight				Duration of experiment, days	Time of experiment	Analysis of bones		Blood phosphorus mg. per 100 cc.	
	Rat No.	Sex	Initial g.	Max. or final g.	Increase %	Mean			H_2O %	Ca on dry wt. %	Inorganic	Total acid-soluble
Exp. 1. *Litter* 33:												
(*a*) Caseinogen heated 36 hours	13	♂	48	118	146	118	55	April–June, 1924	44·5	18·0		
	14*	♂	47	88	91		,,		48	17·0		
	17	♀	49	86	76	88	54		47	18·0		
	18	♀	49	93	90		,,		46	19·1		
								Mean	**46**	**18·0**		
(*b*) Caseinogen heated 72 hours	15	♂	45	89	94	95	55		45	16·1		
	16	♂	48	94	96		,,		45·5	16·3		
	19	♀	49	96	96		54		46·5	17·6		
	20	♀	47	93	98	97	,,		46	18·1		
	21†	♀	46	91	98		,,		50·5	16·6		
								Mean	**47**	**17·0**		
Exp. 2. *Litter* 36:												
(*a*) Caseinogen heated 36 hours	29	♂	45	175	289	218	76	May–July, 1924	41·5	19·0	5·9§	39·4§
	30	♂	47	116	147		,,		45	16·5		
	31	♀	42	110	162		,,		42	13·8		
	32	♀	49	117	139	154	,,		44	17·3		
	33	♀	47	123	162		75		42	18·3		
								Mean	**43**	**17·3**		
(*b*) Caseinogen heated 72 hours	37	♂	50	147	194	171	76		40·0	17·9	5·6§	37·9§
	38	♂	47	117	149		74		44·0	18·0		
	34	♀	45	131	191		76		41	18·9		
	35	♀	42	88	110	155	,,		43	16·0		
	36	♀	48	131	173		,,		40	(lost		
								Mean	**42**	**17·5**		
Exp. 3. *Litter* 38:												
(*a*) Caseinogen heated 36 hours	49	♀	48	127	165	160	84	May–July, 1924	37	22·8	5·5	43·3
	50‡	♀	50	109	118		,,		43	(lost)	4·2	38·3
	51	♀	48	142	196		,,		36	20·5	4·7	36·8
								Mean	**38**	**21·7**	**4·8**	**39·5**
(*b*) Caseinogen heated 72 hours	52	♀	48	110	129	145	84		38	19·4	4·6	38·1
	53	♀	46	122	165		,,		36	20·4	3·9	37·0
	54	♀	49	118	141		,,		39	18·5	4·6	37·7
								Mean	**37**	**19·4**	**4·4**	**37·6**

* Had three fractured ribs. † Had two fractured ribs. ‡ Had four fractured ribs.
§ Determined in pooled blood of group.

Table V.

No. of litter	No. of surviving rats in litter	Date of birth	Age when placed on experimental diet, days (extremes)	Body wt. g. (extremes)	Average body wt. after 21 days on experimental diet, g. ♂	♀	Average maximum wt. attained, g. ♂	♀	% Ca in leg bones, reckoned on dry wt. ♂	♀	Time on experimental diet, days
X	7	16. x. 22	31/34	50/53	69	64	85	105	—	—	—
A	6	30. xi. 22	25	50/53	75	66	99	90	—	—	—
1	7	10. xii. 23	25/26	49/50	69	66	—	—	13·0	14·1	40/41
3	8	11. xii. 23	27	48/51	75	65	—	—	16·3	15·0	38
4	6	14. xii. 23	24	49/50	70	57	—	—	14·8	14·1	38
8	9	4. i. 24	28	50	77	—	93	—	—	—	—
11	9	5. i. 24	27	50	69	—	75	—	—	—	—
17	7	18. i. 24	27	48	68	—	88	—	—	—	—
19	6	25. i. 24	31/33	50	81	65	147	114	—	—	—
33	9	23. iii. 24	28	45/49	67	62	98+	92+	16·8	17·9	54
36	11	1. iv. 24	28/30	42/50	78	67	139	117	17·8	16·9	76
38	13	2. iv. 24	33/36	46/50	77	72	129	119	—	20·3	84
39	13	4. iv. 24	33	50/51	87	79	138	108	19·0	18·8	87
40	10	5. iv. 24	30	50	86	70	130	114	17·3	18·4	89
69	8	21. viii. 24	30	49	98	84	152	118	18·7	20·0	86
73	11	25. viii. 24	28/29	49/52	110	96	139	128	18·3	19·9	86
75	12	2. ix. 24	29	50	87	—	138	—	19·8	—	114
74	13	9. ix. 24	29/30	45/57	87	74	154	113	17·8		94
										18·7	75
76	6	20. ix. 24	28	48	—	79	—	101	18·1	—	96
77	11	3. x. 24	29	44/47	86	75	118+	88+	16·0	17·9	62
80	10	5. x. 24	30/31	49/50	99	99	160+	127+	17·8	—	58
86	9	8. x. 24	27/28	49/51	108	88	146	98	17·3	18·2	64
105	9	19. xii. 24	29/32	45/51	88	75	130+	110+	—	—	—
114	8	31. xii. 24	23/27	47/51	90	69	141+	92+	—	—	—
141	11	13. ii. 25	28/31	48	66	69	88	82	16·7	17·8	73
142	10	14. ii. 25	32/34	47/51	66	66	91	93	16·3	18·7	82
144	7	13. ii. 25	29/31	47/51	90	78	—	—	—	—	—
147	10	18. ii. 25	28	50	—	69	—	—	—	—	—
148	11	20. ii. 25	31/32	46/47	77	73	—	—	—	—	—
177	9	4. iv. 25	24	40/43	58	56	—	—	—	—	—
178	8	7. iv. 25	23	41/45	57	59	—	—	—	—	—
218	9	24. v. 25	26/27	41/43	54	55	—	—	—	—	—
220	7	26. v. 25	28/29	40/46	57	47	—	—	—	—	—
227	11	29. v. 25	31	40/45	60	52	—	67	—	16·7	84
228	9	30. v. 25	26	40/41	51	49	—	—	—	—	—
271	8	20. viii. 25	25/27	40/48	61	51	82	68	14·5	13·7	53/59
272	11	21. viii. 25	26/31	40/46	58	62	88	86	16·6	17·4	50/54
277	8	25. viii. 25	27/29	41/48	69	59	73	81	15·6	17·8	44/50
313	8	19. x. 25	31/33	40/44	61	48	—	—	—	—	—
318	11	20. x. 25	31/34	41/47	66	61	—	—	—	—	—
324	9	22. x. 25	29/30	43/46	63	—	—	—	—	—	—
316	6	22. x. 25	29	45	—	60	—	—	—	—	—
319	9	26. x. 25	24	43/44	71	—	—	—	—	—	—
322	9	28. x. 25	23/30	43/46	61	59	—	—	—	—	—
323	10	30. x. 25	25	43	67	—	—	—	—	—	—
325	9	30. x. 25	28/29	44/45	62	—	—	—	—	—	—

Reserves of fat-soluble vitamins in the experimental animals.

The reserves of fat-soluble vitamins possessed by the young rats at the beginning of the experimental period are known to affect greatly their behaviour when placed later upon diets deficient in these vitamins, and the diet of our breeding stock was, therefore, carefully scrutinised. The accompanying paper [Smith and Chick, 1926] (published in conjunction with Miss Henderson Smith, who is in control of the breeding of rats designed for nutritional work in this Institute), deals with the question of diet and of the

technique which has been evolved for maintaining a supply of young rats which shall be reasonably constant as regards their reserves of fat-soluble vitamins. The diet of the breeding stock, which contains a fair proportion of whole cereals and of fresh green food, is moderately rich in vitamin A, but comparatively poor in vitamin D, except in summer, when the ration of fresh cow's milk contains appreciable amounts of the latter vitamin [see Luce, 1924, 1, 2; Boas and Chick, 1924]. Luce [1924, 1] found the vitamin A content of summer "pasture fed" cow's milk to be about 2–3 times that of milk from the same cow when fed on dry fodder in a dark stall in winter. The relative increase in anti-rachitic value (vitamin D) was somewhat greater viz. 4–5 times. When the whole diet of the breeding stock is taken into account, the change in total vitamin A due to the seasonal variation in the vitamin A content of the milk ration will probably be small in comparison with the change in the total vitamin D.

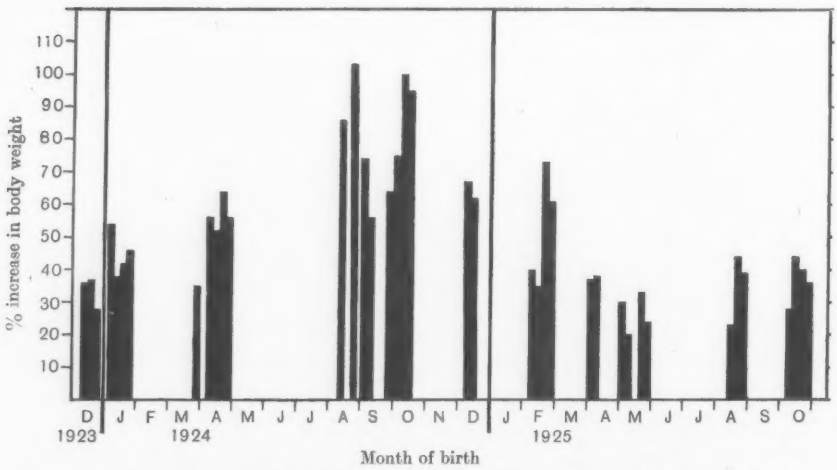

Fig. 1. Influence of the month of birth upon the average percentage increase in body weight shown by young rats (40–50 g. original weight) after 21 days upon diets deprived of fat-soluble vitamins.

It was conceivable, therefore, that variations in the nutritional value of the fresh milk supplied to the lactating mothers and to the young up to the time of experiment might explain the irregularity which was being investigated. Accordingly, arrangements were made to provide the mothers after the birth of the young with dried milk prepared from winter milk, enough being prepared in March 1925 to last over the summer and autumn. The lower portion of Table V from litter 177 downwards and the right-hand section of Fig. 1 summarise our experience since the alteration was made. The average weight after 21 days on the experimental diets devoid of fat-soluble vitamins, as well as the average maximum weight attained, remain roughly constant for

animals born during the summer and autumn of 1925, although the young animals are seen to possess lower vitamin reserves than previously, judged by the amount of growth.

Table VI. *Variations shown by young rats after 4 weeks on McCollum's low P rickets-producing diet, No. 3143.*

All rats bred in the Institute on stock diet. Egg-yolk included in stock diet from Sept. 10 to Dec. 10, 1924. Full cream dried milk substituted for fresh milk from Dec. 2, 1924 to March 6, 1925.

Litter No.	Date of birth	No. of rats in litter	No. of rat	Sex	Age when placed on experimental diet, days	Body weight: Initial g.	Body weight: Final g.	Body weight: Increase %	Calcium content of long bones, % calculated on dry wt. of bone	Degree of rickets judged by histology of rib-junctions	Notes
78	2. x. 24	11	30	♂	32	50	78	56	13·1	Moderate	Mother received egg-yolk on one or two occasions during period of lactation (21 days)
			37	♀	38	49	82	67	15·1	,,	
87	10. x. 24	5	41	♂	25	51	90	76	16·0	Slight	,, ,,
111	19. xii. 24	8	70	♂	33	48	92	92	14·3	Moderate to severe	
			68	♀	34	50	86	72	12·3	Severe	
113	29. xii. 24	6	82	♂	28	52	75	44	12·9	Moderate to severe	
155	2. iii. 25	11	337	♂	29	48	76	58	12·2	Severe	
			338	♀	31	44	81	84	13·8	,,	
166	11. iii. 25	11	342	♂	36	47	61	37	17·0	Slight	Mother received dried March milk during lactation period
165	14. iii. 25	11	354	♀	33	43	68	58	15·4	Moderate	,, ,,
232	4. vi. 25	8	410	♂	26	48	89	85	9·8	Severe	,, ,,
			414	♀	26	46	75	63	12·4	,,	,, ,,
233	11. vi. 25	9	420	♀	30	46	62	35	11·8	,,	,, ,,
289	14. ix. 25	11	454	♂	33	45	63	40	10·7	,,	,, ,,
			458	♀	33	44	63	43	11·9	,,	,, ,,
301	18. ix. 25	10	228	♂	29	44	67	52	10·2	,,	,, ,,
			230	♀	29	46	59	28	10·2	,,	,, ,,
302	30. ix. 25	7	462	♂	26	44	62	41	9·9	,,	,, ,,
			465	♀	26	45	54	20	10·5	,,	,, ,,

It is our belief that this is due chiefly to a diminution of the vitamin D in the mothers' diet, for the substitution of dried winter milk for the fresh milk previously supplied in summer and autumn had reduced the intake of this vitamin, while affecting less seriously the total intake of vitamin A. It so happened that the young born during the previous winter had received an unusually large endowment of vitamin D, due to an unwise addition of egg-yolk to the diet of the breeding stock during the autumn (Sept.–Dec. 1924). Egg-yolk is a rich source of vitamin A and also very rich in anti-rachitic properties [Hess, 1923; Casparis, Shipley and Kramer, 1923; Hess and Weinstock, 1924], and this addition endowed the young with such large reserves of vitamins A and D that they proved useless for the assay of either vitamin. When placed at 40–50 g. weight upon the diet deprived of fat-soluble vitamins, they often grew normally for several weeks (*e.g.* Table V, litters 74, 80, 105) and developed well-calcified bones. Upon McCollum's No. 3143 low phosphorus, rickets-producing diet an abnormal amount of growth was made

in many cases, and at the end of 4 weeks the macroscopic signs of rickets were slight or altogether absent. The degree of rickets diagnosed histologically was often slight or moderate in place of the severe type usually produced, whilst the calcium content of the bones varied from 12–17 % in place of the 9–13 % usually found [see also Luce, 1924, 1, 2]. In Table VI are collected the results obtained on McCollum's diet with litters born from Oct. 1924 to Sept. 1925. It will be seen that after March 1925, when the mothers received only dried winter milk, a satisfactory degree of constancy is attained in the behaviour of the young.

Summary.

1. An important source of error in the biological method of estimating the fat-soluble vitamins A and D in various foodstuffs consists in variation in the reserves of these two vitamins which may be present in the young rats used for experiment. These reserves depend on the diet upon which the young rats are reared.

2. With the diets ordinarily used in breeding rats for such work, the reserves of vitamin D are usually low in comparison with those of vitamin A. Great irregularity s caused if materials rich in vitamin D are at times included in the diet. Substances to be avoided are egg-yolk and cod-liver oil. Summer, "pasture fed," cow's milk should be used sparingly.

3. The degree of illumination of the test rats while under experiment (within the limits attained by ordinary daylight passing through glass windows at different seasons of the year) is of slight importance.

4. If the hypothesis of Steenbock and his co-workers be accepted that the anti-rachitic vitamin D is essential for growth equally with vitamin A, and that lack of one in a diet may limit growth in presence of adequate amounts of the other and of the remaining essential factors—it follows that much previous work concerned with the distribution and estimation of vitamin A needs revision.

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XXI. MAINTENANCE OF A STANDARDISED BREED OF YOUNG RATS FOR WORK UPON FAT-SOLUBLE VITAMINS, WITH PARTICULAR REFERENCE TO THE ENDOWMENT OF THE OFFSPRING.

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In an accompanying paper [Chick, 1926] one of the writers discusses some sources of error in the technique usually adopted in the investigations of fat-soluble vitamins by the biological method. The conclusion reached, based on the collective experience of the workers in this Institute over a period of several years, is that one, perhaps the most important, disturbing factor is the variation in the state of nutrition of the young rats employed in the tests; in other words variation in the reserves of these vitamins possessed by the young animals at the beginning of the experimental period.

In order to attain a reasonable degree of accuracy in the estimation of fat-soluble vitamins in different foodstuffs and the comparison of one such material with another, it is essential to have at one's command a standard young rat, which can be depended upon to react subsequently in a reasonably constant manner to the same type and degree of deficiency. Special care is needed in breeding such animals if this ideal is to be realised, and in the Lister Institute, as elsewhere, the workers in this field have found it necessary to supervise directly the breeding of the rats destined for their own use.

During several years' experience the greatest difficulty has been experienced in obtaining the constancy required for accurate work. At the present time, however, the difficulty appears to be overcome to a large extent and the following account of our methods is published here in the hope that it may prove useful to others who may be confronted with the same problems.

The amount and nature of the reserves with which the experimental animals are equipped at the beginning of the observation is more important in nutritional experiments of long duration than has been generally recognised. This is especially so when the subsequent work involves diets deficient in fat-soluble vitamins. For even very young animals possess reserve stores of these factors to a surprisingly variable degree, depending on the diet of the mother before and during pregnancy, and during the period of lactation.

Korenchevsky [1921, 1922] and Korenchevsky and Carr [1923, 1924, 1925] have shown that the effects produced upon young rats receiving diets deficient in fat-soluble vitamins will be widely divergent, in respect both to skeletal disorders and to the degree of growth in weight, according as the diet of the mother during pregnancy and lactation has been defective or generous in these vitamins as well as in the salts of calcium. When, for example, the mothers received cod-liver oil and extra calcium during lactation, the young rats after 30 days' independent existence on a diet deprived of all fat-soluble vitamins, were found to possess skeletons which might be regarded as normal [Korenchevsky and Carr, 1923]. Cramer [1922] drew attention to the remarkable difference in growth exhibited by two breeds of young rats after being maintained for several weeks upon diets devoid of fat-soluble vitamins. There was a difference between the diets on which the two groups had been reared; in one case the mothers had received a diet consisting mainly of cereals and in the other there had been additions of cod-liver oil. Hess, Weinstock and Tolstoi [1923] noticed that certain young rats bred outside the laboratory could maintain a normal condition of the skeleton after receiving a standard, low phosphorus, rickets-producing diet for several weeks. Here also the cause was traced to a superior diet enjoyed by the breeding stock. For, if the mothers at the end of pregnancy were brought into the laboratory and thereafter received the usual laboratory diet, the young rats proved to have the usual susceptibility, when placed later upon the experimental diets. These authors explain the differences, resulting from the use during lactation of diets richer and poorer respectively in anti-rachitic foods, as due to the food taken directly by the young during the last days of the period, when they are capable of feeding themselves [Hess and Weinstock, 1924, 1, 2]. Korenchevsky and Carr [1923], on the other hand, on the basis of experiments in which the mother alone had access to the special anti-rachitic food (cod-liver oil) provided showed that the protective substances can be transferred to the young with the mother's milk.

It is therefore clear that in breeding rats for experimental work upon fat-soluble vitamins, the diet, whilst possessing an abundance of other essential elements—protein of good quality, mineral salts and vitamins B and C—should contain a constant, but not excessive, amount of both vitamin A and vitamin D. For the above researches give abundant proof that young rats endowed with unduly large reserves of these substances will later on prove insensitive to the deficiencies in diet which are the object of study.

Steenbock and Nelson [1923] were the first to point out the necessity of considering the reserves of these two vitamins, A and D, separately (see also Chick, 1926]. The hypothesis of these workers is that the degree of growth subsequently manifested by the young when placed upon a diet deprived of *both* factors, is dependent jointly upon the reserves of *both*, either factor alone being unable to promote growth, though capable of maintaining weight for a time, in the absence of the other.

This theory, for which there is much experimental support, has not only an important bearing upon the methods usually employed in work with fat-soluble vitamins, dealt with in detail in the preceding paper, but has also focussed attention upon the importance of the relative amount of vitamins A and D in the diet upon which the experimental rats are reared. Steenbock and Nelson [1923] called attention to the fact that young rats placed upon diets deficient in fat-soluble vitamins may cease to grow several weeks before the onset of the ophthalmia which is the characteristic sign of vitamin A deficiency. From this circumstance they conclude that it is usual for such young rats to possess more vitamin A in reserve than anti-rachitic vitamin D. Although in our experience the first signs of ophthalmia have usually been detected before cessation of growth in weight, we agree with the conclusion of Steenbock and Nelson, and in the light of later knowledge we realise that the more unexpected and unexplained irregularities have occurred when we have unintentionally enriched the breeding diet with vitamin D, as provided, for example, in summer cow's milk or egg-yolk. Steenbock and Black [1925] encountered similar disturbing effects when unwisely submitting their breeding stock to irradiation with ultra-violet light as a therapeutic measure when the young were not thriving in a normal manner.

General management of the breeding stock.

We have worked hitherto, almost exclusively, with the black and white pied variety of the Norwegian rat, but the albino type has been found equally suitable by many workers, notably by Donaldson [1924] at the Wistar Institute. Our original stock was obtained from the department of physiology at King's College for Women by the courtesy of Dr Gladys Hartwell. Although inbreeding is our custom, it has been our habit about once a year to obtain some young rats from outside to refresh the stock, and these have usually been obtained from Dr Hartwell.

The rats are kept in a laboratory with north aspect and the temperature maintained between 65° and 70°. Breeding is not permitted before the female rats are about four months old. After that time about 4 to 8 females are placed with 1 to 2 bucks in large zinc wire cages of dimensions 20 inches by 30 inches by 30 inches high. The cages have loose wire bottoms and rest upon large trays filled with peat. The rats are provided with a bedding of wood wool, which is changed every day, whilst the cages and trays are scoured thoroughly twice weekly.

Pregnant females are removed from the general cage 7 to 10 days before the birth of the young and are kept separately in cages of dimensions 20 inches by 10 inches by 7 inches high. Here the young family remain together with the mother for 21 days which is the usual interval before weaning. The mother is then removed and enjoys 2 weeks' rest before breeding is again permitted. As a rule not more than the first three litters from any female are used; our experience in this matter coincides with that of the Wistar

Institute that fourth and subsequent litters are too small and irregular for this type of work. We have found the greatest number of large and healthy litters to be born from April to September. In our experience the months of October, November and December, especially the last two, have proved bad for breeding. Few litters are born, these are frequently feeble and the mothers appear to lack a sufficient milk supply. After the turn of the year things gradually improve as spring approaches. Excluding the month of November the average number of young rats reared in the first and second litters cast by the females of our breeding stock is between 8 and 9, a figure considerably higher than that given by Donaldson [1924], viz. 6 to 7 per litter for first and second litters of the tame albino, and 5 to 6 per litter for the wild Norwegian rat.

Diet.

The diet we have found satisfactory consists of cow's milk, brown or white bread, or rice or oats or other whole cereals and seeds, with fresh raw vegetables, carrot, turnip, cabbage or spinach. Marmite is mixed with the food every day to provide an abundance of vitamin B and raw lean meat is given about twice a week. Within the above choice of foods the ration is varied daily so that the animals get plenty of change. This we have found a necessary measure to encourage a good appetite and an adequate intake of food, which is particularly necessary in the case of the female rats from which litters are expected. During the last few days of pregnancy and during the period of lactation the mother receives only milk, cereals and raw vegetables, with marmite added. Lean meat has occasionally been given when a tendency has been suspected to eat the young, which may occur at the times of the year which are unfavourable for breeding.

Although this breeding diet was maintained constant throughout the year, it had been noticed that the young rats born in winter and spring were markedly inferior to those born in summer and autumn. This was shown by a lessened capacity to grow and produce a well calcified skeleton, when placed on diets devoid of fat-soluble vitamins. After investigating many other possible sources of this irregularity, the cause was at length traced to the diet of the breeding stock and found to consist in the variations in the nutritive value of the daily milk ration at different seasons of the year. Luce [1924] and Boas and Chick [1924] have demonstrated the superiority as regards vitamins A and D of summer, "pasture fed" cow's milk compared with that obtained from cows kept in stalls and receiving dry fodder (hay, cereals and roots) in winter. The summer pasture feeding seems to affect the value of the ordinary milk supplied in London from about May to October or November, depending on the season; the lowest vitamin content occurs in February or March.

In order to avoid the irregularity due to this variation, it was determined to abandon the use of fresh cow's milk and to achieve constancy by substituting

a reconstituted dried milk, prepared in quantity from the ordinary milk supply of early spring. Winter rather than summer milk was chosen as it was considered desirable to avoid any possibility of excessive reserves in the young rats, which would render them insensitive to deficiencies of diet encountered later on. Accordingly, in March 1925, a supply of fresh milk sufficient to last over the summer and autumn was kindly dried for us by Glaxo Ltd. At first this reconstituted milk was supplied indiscriminately to the whole breeding stock, but, after making the change, the general nutritive condition was found to be lowered dangerously near the margin at which the young could be reared successfully. Fresh milk was therefore restored to the diet of the general stock and the dried winter milk reserved for mothers during the last days of pregnancy and the period of lactation. It is also fed to the young during the interval elapsing after weaning and before being used for experiment. This arrangement has proved successful in securing a reasonable degree of constancy in the young rats as regards their reserve supply of fat-soluble vitamins [see also Chick, 1926, Fig. 1 and Table V].

As is mentioned above, the months of November and December offer special difficulties in the breeding of rats, for some unknown reason. In our need for young litters at all times of the year we are probably guilty of forcing the rats somewhat in the matter of breeding, and at the less favourable seasons it is necessary to tempt the appetite of the mother with changes of food if the young are to be reared successfully. A ration of fresh milk is occasionally given at discretion and extra lean meat.

It is probable that the seasonal irregularity noticed previously in our breed was due to variation in the supply of anti-rachitic vitamin D rather than of vitamin A. The stock diet, consisting of cow's milk, whole cereals and fresh green vegetables, is moderately rich in the latter vitamin, even in winter, when the supply of the anti-rachitic vitamin D will be very low.

This view was confirmed in an interesting, if somewhat disastrous, manner in the autumn of 1924. Large quantities of fresh egg-white were being used in the Institute in connection with other researches, and in the interests of economy the yolks were used in the diet of the rat breeding stock and in some cases added to the ration of lactating mothers whose litters did not appear to be thriving. The effect of adding a substance so high in anti-rachitic value [Hess, 1923; Casparis, Shipley and Kramer, 1923; Hess and Weinstock 1924, 1, 2] was not contemplated and the results, though admirable from the standpoint of the mother and the young litters, proved disastrous from the point of view of the subsequent experiments. The young rats, placed at 50 g. weight upon McCollum's No. 3143 rickets-producing diet [McCollum, Simmonds, Shipley and Park, 1921], showed only a slight or moderate degree of rickets after 4 weeks upon the diet, in place of the usual severe type [see Chick, 1926, Table VI]. The calcium content of the bones was also at an unusually high level and varied from 12 to 17 % (reckoned on the dry weight of the bones) instead of the usual 9 to 13 % [see also Luce, 1924].

The experiments had been arranged to determine the anti-rachitic value of samples of milk, which were added to the diet in a range of daily doses, but they gave unreliable results and could not be used in comparison with the results of other tests in which the experimental animals had been less richly endowed with anti-rachitic vitamin and had reacted to the defective diet in a more normal manner. This marked enrichment of the breeding diet with vitamin D appeared to affect the reserves possessed even by the young rats born several weeks after its use had been discontinued.

Summary.

1. A description is given of the diet and general management found satisfactory in breeding rats for nutritional work upon fat-soluble vitamins.

2. The diet used, cereals, fresh vegetables and milk, is moderately rich in vitamin A but poorer in vitamin D, except in summer, when fresh pasture milk contains a much increased amount of this vitamin. This circumstance has been the cause of a disturbing amount of variation in the nutritive condition of the young at different seasons of the year.

3. The expedient of replacing fresh milk in the diet of the pregnant and lactating mothers by dried milk prepared from the winter milk supply has proved successful in lessening the degree of irregularity among the young.

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XXII. THE ANTI-RACHITIC VALUE OF FRESH SPINACH.

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Introduction.

Fresh green leaves form the entire diet of many higher animals and may therefore be supposed to contain the various factors necessary for complete nutrition. Experimental proof is forthcoming for the presence of many of these, including vitamins A and C. As regards the presence of the anti-rachitic vitamin D, however, the evidence is conflicting. The majority of workers who have investigated the point have found that green leaves, whilst rich in the fat-soluble vitamin A, are devoid of anti-rachitic properties, even when administered in large doses. This constituted one of the most convincing pieces of evidence in favour of the view advanced by McCollum and his co-workers [1922] that vitamin A and the anti-rachitic substance found associated with certain fats are not one substance, but two separate entities.

The previous experimental work concerned with the detection and evaluation of anti-rachitic properties has, however, been almost exclusively carried out with the aid of diets deficient in phosphorus, but rickets may develop in children reared on cow's milk, which affords an abundant supply of calcium and phosphorus. The puppy behaves similarly, as has been demonstrated by Mellanby and others. But in order to induce rickets with certainty in the young rat—the animal which has been largely adopted in the experimental study of rickets, because it is in many respects the more convenient—it has been found necessary to use diets deficient in calcium or phosphorus as well as in other factors. The famous rickets-producing diets of McCollum and his co-workers [1921], of Sherman and Pappenheimer [1921], and of Osborne, Mendel and Park [1923] are instances of diets short, but not deprived, of fat-soluble vitamins and at the same time very low in phosphorus.

To deprive the growing animal of phosphorus, however, is to complicate the issue by setting up a severe competition for this element between the bones on the one hand and the various soft tissues on the other. The situation is somewhat otherwise in the case of calcium. The skeleton of the rat contains calcium and phosphorus in the ratio, approximately, of 2 to 1, and whereas all but about 7 % of the total calcium contained in the body of rats weighing from 70 to 100 g. is in the bones [Boas and Chick, 1924], the soft tissues

account for nearly 30 % of the total phosphorus. This striking quantitative difference suggests that to deprive the growing animal of phosphorus might cause a more severe competition for this element between the bones and soft tissues than would occur even in case of a shortage of calcium, for which a comparatively small demand is made by the soft tissues.

Using diets deficient in phosphorus, as well as in fat-soluble vitamins, anti-rachitic influences have been demonstrated in sunlight [Hess, Unger and Pappenheimer, 1921] and ultra-violet radiation [Powers, Park, Shipley, McCollum and Simmonds, 1922], in cod-liver oil and some other fats [McCollum, Simmonds, Becker and Shipley, 1922] and in egg-yolk [Hess, 1923]. Many other foodstuffs, on the other hand, have been denied anti-rachitic value, and among them fresh green vegetables. McClendon and Shuck [1923] were unable to detect appreciable anti-rachitic properties in dried spinach leaves, even when consumed in amounts equivalent to 75 % of fresh leaves in the total diet, whereas amounts equal to 0·1 % showed a definite retardation of the onset of xerophthalmia. Goldblatt and Zilva [1923] obtained a similar negative result with a daily dose of 3·0 g. fresh spinach given as supplement to McCollum's No. 3143 diet [McCollum, Simmonds, Shipley and Park, 1921], a diet deficient in phosphorus as well as in fat-soluble vitamins. At the same time the minimal daily dose of spinach found necessary to stimulate growth, which had ceased upon a diet deficient only in fat-soluble vitamins, was found to be between 0·1 and 0·25 g. Zucker and Barnett [1923] could detect no anti-rachitic value in extracts of spinach leaves, in the preparation of which alcohol, acetone and ether were employed, but their experiments were also made with diets very low in phosphorus.

Hess and Weinstock [1924, 1925] found no anti-rachitic properties in fresh lettuce leaves. But the leaves, whether grown in the light or etiolated by growth in the dark, acquired anti-rachitic potency if irradiated with the mercury vapour lamp for $\frac{1}{2}$–1 hour, which potency was not lost after three days' storage in the dark. The doses given were however very large, 10 g. per diem for young rats of 30–40 g. weight, and the growth was very slight.

On the other hand, Hart, Steenbock, Hoppert and Humphrey [1922] and Gaessler and McCandish [1923], working with milch cows, found that the addition of fresh or dried alfalfa to the diet had a beneficial influence upon the calcium balance of the cow, provided the alfalfa had not been stored too long. Recently Shipley, Kinney and McCollum [1924], working with rats, have also found definite anti-rachitic value in dried alfalfa, when administered in the form of extracts made with ether, alcohol and acetone. They used amounts in the diet equivalent to 25 %, reckoned as fresh tissue; they failed however, to detect any evidence of anti-rachitic action in case of cabbage, spinach and other vegetables. McClendon [1922] states that rickets can be prevented on a low phosphorus diet by means of spinach if 40 % is included.

Luce [1924] found that milk from a cow on pasture in summer had a high anti-rachitic value compared with that of milk given by the same cow when

receiving dry fodder in a dark stall in winter and came to the conclusion that the increase of vitamin D in the milk was due partly to the fresh green food and partly to the insolation enjoyed by the cow. The criterion adopted was the power of the milk to prevent rickets in young rats upon McCollum's No. 3143, low phosphorus, rickets-producing diet; and in this case, as in other researches employing diets of this character, the criticism is justified that the anti-rachitic effect, at least in part, may be due to the extra phosphorus received with the rations of milk. Since the protective daily dose of the "pasture-fed" milk lay between 2 and 5 cc., whilst from 15 to 20 cc. daily of the winter milk was required, the salt content of both milks being approximately the same, it is reasonable to assume a considerable superiority in the former of anti-rachitic properties.

In view of these somewhat conflicting results and of the importance of the subject, it seemed worth while to make further attempts to ascertain whether any anti-rachitic value could be demonstrated in fresh green leaves of other plants than alfalfa and in so doing to abandon altogether the use of diets deficient in phosphorus. The fresh leaves of the "prickly seeded" variety of spinach (*Spinacea oleracea*) were chosen because this variety of spinach can be grown in this country at most times of the year and is readily eaten by young rats; under favourable conditions it is possible to administer as much as 2 to 3 g. daily.

Methods.

Young rats, about three weeks old and 25 to 35 g. in weight, were placed upon diets devoid of fat-soluble vitamins, but adequate as regards salts of calcium and phosphorus. The behaviour of rats receiving various daily rations of fresh spinach leaves was compared with that of control animals receiving the basal diet without addition, during a period of about six weeks. The increase in body weight during the period was taken as an indication of the amount of vitamin A contained in the spinach (possibly of the vitamin A combined with the vitamin D), whilst the degree of calcification of the bones at the end of the time provided a measure of the calcium-depositing power of the ration (vitamin D content). In most of the experiments comparison was also made with one or more animals from the same litter, which animals received cod-liver oil, or irradiation with ultra-violet light, or a ration of irradiated spinach.

Basal diet. The experimental diet, devoid of fat-soluble vitamins, which is usually employed in this Institute has the following composition:

Inactivated caseinogen 100 g., wheaten starch 250 g., cotton-seed oil 75 g., marmite 25 g., lemon juice 25 cc., salt mixture[1] 25 g. This diet which is known as "F" diet contains 0·4 % Ca and 0·9 % P reckoned on the dry weight and

[1] The salt mixture was the following:

NaCl	43 g.	$CaH_4(PO_4)_2 . H_2O$ ...	136 g.
$MgSO_4 . 7H_2O$	137	Ca lactate ($5H_2O$) ...	327
$NaH_2PO_4 . H_2O$ ...	87	Ferric citrate	30
K_2HPO_4	240		

was used for the tests in Exps. 1, 2, 5. These amounts of calcium and phosphorus were, however, thought to be rather in excess of those occurring in more natural food and a second diet was devised, containing smaller proportions of Ca and P, viz.:—"F.R. diet" used in Exps. 3 and 4. In arranging this diet, the food eaten by the stock animals in this Institute was taken as a guide. This food consists mainly of bread, brown and white, cow's milk and fresh carrot and cabbage, with occasional additions of marmite and raw lean meat. It was estimated that a normal young rat of 70 g. weight would consume daily about 2 g. cabbage, 10 cc. milk, 15 g. bread. Such a diet would contain about 0·23 % Ca and 0·32 % P, reckoned on the dry weight of the diet.

	g.	Dry wt., g.	Ca, g.*	P, g.*
Cabbage	2	0·2	0·0023	0·0022
Milk	10	1·0	0·011	0·0083
Brown bread	15	9·0	0·009	0·022
Total	27	10·2	0·023	0·032

* Figures taken from Schall-Heisler [1917].

The following diet was prepared to reproduce the above diet of cabbage, bread and milk as regards the inorganic constituents: inactivated caseinogen 100 g., wheaten starch 250 g., hardened cotton seed oil 75 g., salt mixture ($MgCO_3$ 14 parts, $NaHCO_3$ 53 parts, NaCl 13 parts) 9 g., ferric citrate 0·4 g., calcium carbonate 1·5 g., K_2HPO_4 0·8 g., distilled water 300 cc. Direct estimations made on different batches of the diet, for which we are indebted to Dr M. Boas, gave the following mean values:

	Water	...	45 %	on the fresh weight
	Ca	...	0·15 %	
	P	...	0·20 %	
and	Ca	...	0·27 %	on the dry weight.
	P	...	0·37 %	

About one-half of the total calcium is derived from the calcium carbonate and about one-half from the caseinogen. Of the phosphorus also about one-half is accounted for by the caseinogen and the other half by the potassium phosphate and other constituents. So that, as in the natural diet which was taken as a model, a large proportion of the salts both of calcium and of phosphorus are presented in the form in which they exist in milk.

The rats were considerably younger than those usually employed in such tests, this being arranged to secure that the vitamin reserves in the test animals should be small. There is usually a check in growth and a tendency to diarrhoea when young rats, immediately after weaning, are placed upon artificial diets devoid of fat-soluble vitamins. To tide them over the change it is a good plan to provide bedding of purified cotton wool and to give a ration of dried cows' milk of low vitamin content for a day or two. In the earlier experiments control rats or those receiving the same additional rations were placed together, 2 or 3 in the same cage, but in the end it was found

more satisfactory to keep each animal separate after the first few days of the experiment. The looseness of stools was found to be more persistent on the F.R. than on the F diet, owing to the lower proportion of calcium salts in the former. In Exp. 4 the rats receiving cod-liver oil and the irradiated control animals were so severely affected that many had to be killed before the end of the experiment and thereafter only the F diet was used. Even so, irradiated animals and those receiving irradiated rations of spinach underwent a period of mild diarrhoea at the beginning of the observation; this, however, did not last more than 1 to 2 weeks.

All the animals except those receiving irradiation were kept in a poorly illuminated laboratory, screened from direct daylight. At the end of the experiment the rats were killed, the junctions of the 6th and 7th ribs on the right side were preserved for histological examination and the leg bones (femora, tibiae and fibulae) dissected out for chemical analysis. The water content was estimated by the loss of weight on drying for 24 hours at 105° to 110° and the fat by the loss of weight on boiling in alcohol for about 16 hours followed by extraction with ether in a Soxhlet apparatus for 24 hours. If the bones when fresh are split with a sharp scalpel, it is safe to reduce the time of the ether extraction to about 8 hours.

In Exps. 1, 2, 3 calcium was determined in the dried or fat-extracted bones by the method of Aron and Sebauer [1910]; in the later experiments (4 and 5) the fat-extracted bones were ashed in porcelain crucibles and the calcium calculated as 36 % of the weight of the ash. This figure was arrived at as the result of a series of analyses of similar bones in which the calcium was estimated directly in the ash by the method described by Boas [1924] based on that of McCrudden[1] [1909, 1911]. The mean figure found for 12 determinations was 35·94 % of the ash, the extremes being 34·85 and 37·18 %.

The histological diagnosis was kindly undertaken by Prof. V. Korenchevsky, who has added a note to this paper, explaining the criteria adopted.

Experiments.

Exps. 1 *and* 2 (*Tables I and II*). Table I contains the details of Exp. 1 made with a litter of 12 young rats divided into four groups of three. One group received the basal diet only, whilst three similar groups consumed respectively daily rations of 0·1, 0·5 and 1·0 g. fresh raw spinach leaves in addition. The observation lasted 50 days. The rats which received the 0·1 g. spinach ration had a mean calcium content of the bones of 16·4 %, as compared with 14·1 % in control animals, both figures being reckoned on the dry weight of the bones (femur, tibia and fibula). The daily rations of 0·5

[1] In a series of estimations, in which the Ca content was determined separately in the two leg-bones of the same animal by the two methods respectively, the figure obtained by McCrudden's method was found to be on an average 3 % lower than that given by the method of Aron and Sebauer. The calcium determined by the latter method was found to be equal to 37·14 % of the weight of the ash.

and 1·0 g. spinach raised the Ca content to 16·5 and 16·8 % respectively. At the same time the growth of these rats and their general condition were much in advance of those of the control animals. The concentration of inorganic phosphorus in the blood was within normal limits in all cases.

Table I. Exp. 1. *Influence of fresh spinach upon calcium deposition in bones of young rats maintained on* F *diet deficient in fat-soluble vitamins* (Ca = 0·4 %, P = 0·9 %, *reckoned on dry weight of diet*).

Litter of 12 young rats, 21 days old at beginning of experiment. Duration of experiment = 50 days. March–May 1924.

Spinach daily ration g.	Rat No.	Sex	Body weight: Initial g.	Body weight: Max. or final g.	Body weight: Increase g.	Increase % (males only)	Leg bones, femur, tibia and fibula: H_2O %	Leg bones, femur, tibia and fibula: Ca % (reckoned on dry wt.)	Average intake of basal diet g. daily	Blood P mg. per 100 cc.: Inorganic	Blood P mg. per 100 cc.: Total acid-soluble
0·0	1	♂	34	65	31	74	48·9	13·6	8·0	9·8	40
	2	♂	35	55	20		42·7	13·0			
	3	♀	38	69	31	—	45·4	15·7			
						Mean	**45·7**	**14·1**			
0·1	4	♀	35	55	20	—	51·2	16·0	9·0	8·4	36
	5	♂	37	76	39	122	47·0	17·35			
	6	♂	35	84	49		50·1	15·7			
						Mean	**49·4**	**16·4**			
0·5	7	♀	33	52	18	—	51·5	15·6	9–10	8·4	36
	8	♀	33	71	38	—	47·0	16·4			
	9	♂	36	96	60	167	49·1	17·4	10–14		
						Mean	**49·2**	**16·5**			
1·0	10	♀	34	74	40	—	47·2	16·9	10–11	8·3	42
	11	♂	38	129	91	200	49·6	18·0	10–17		
	12	♂	36	93	57		49·0	15·6	10–12		
						Mean	**48·6**	**16·8**			

Table II. Exp. 2. *Influence of a small daily ration of fresh spinach in inducing calcium deposition in bones of young rats maintained on* F *diet.*

Rats 19 days old at beginning of experiment. Duration of experiment = 43 days. April–July 1924.

Spinach daily ration g.	Rat No.	Sex	Body weight: Initial g.	Body weight: Max. or final g.	Body weight: Increase g.	Mean % increase	Leg bones. Ca % reckoned on dry wt.	Histology of rib-junctions
0·0	22	♂	29	56	27	109	11·9	Osteoporosis and slight rickets
	23	♂	29	65	36		14·9	Infraction and osteoporosis
	24	♀	30	54	24	88	15·9	Osteoporosis
	25	♀	27	53	26		15·3	Osteoporosis
						Mean	**14·5**	
0·1	26	♂	29	75	46	167	15·5	Osteoporosis and very slight rickets
	27	♂	31	85	54		16·5	Osteoporosis
	28	♀	26	46	20	77	15·6	Osteoporosis and slight rickets
						Mean	**15·9**	

The details of a second experiment in which a litter of seven young rats was employed are set forth in Table II. This experiment lasted 43 days. At the end of this period three rats, which consumed a daily ration of 0·1 g.

Table III. Exp. 3. *Diet F.R.* (Ca = 0·3 %; P = 0·4 %; *reckoned on the dry weight.*) *Early spring spinach. Feb.–April* 1925.

Litter	Rat no.	Sex	Dose, daily	Duration of experiment days	Body weight Initial g.	Body weight % increase	Analysis of bones, calculated on fresh weight, % H_2O	Fat	Ash*	Residue	A/R	% Ca on dry wt.	% Ca on extr. dry wt.	Ophthalmia	Histology of rib-junctions	Remarks
140	127	♂	0, Control	50	27	81	Not determined					16·2	17·3	Very severe	Osteoporosis	Growth poor
	131	♀	0, Control	45	24	87	56·8	0·73	19·36	23·15	0·836	16·6	16·85	Severe	Osteoporosis	Growth poor. Died on 45th day
	129	♂	Spinach, 0·1 g.	51	29	203	56·0	5·18	17·58	21·19	0·830	14·8	16·8	Slight	Slight rickets	Growth subnormal
	132	♀	,, 0·5 g.	51	22	245	55·7	3·55	18·90	21·85	0·965	15·8	17·2	,,	Very slight rickets	,,
	130	♂	,, 2·6 g. (average)	51	27	289	56·4	3·65	18·01	21·91	0·922	15·3	16·7	Very slight	,,	Growth normal. P.M. 4 ribs fractured, fibula fractured
	128	♂	Cod-liver oil, 1 drop	51	27	263	48·6	3·73	26·92	20·73	1·298	19·4	20·9	Nearly normal	Normal	Growth subnormal, but fair
146	133	♂	0, Control	39	35	103	51·6	8·42	18·17	21·82	0·833	13·9	16·8	Very slight	Osteoporosis	Growth poor
	139	♂	Spinach, 2·2 g. (average)	37	30	167	56·1	3·89	16·63	23·39	0·711	14·0	15·4	Slight	Osteoporosis and slight rickets	Growth nearly normal. P.M. 1 rib fractured
	135	♂	Cod-liver oil, 1 drop	39	32	231	48·3	5·68	24·78	21·21	1·168	17·8	20·0	Normal	Nearly normal	Normal growth
Animals irradiated:																
	134	♀	0	39	32	150	46·0	6·81	26·78	20·37	1·315	18·4	21·0	Slight	Some osteoporosis	Growth fair
	138	♀	Spinach, 0·5 g.	36	31	123	47·7	5·89	24·60	21·82	1·127	17·4	19·6	? Very slight	Nearly normal, some osteoporosis	Growth good at first. Died on 26th day, after an accident; animal lost appetite

* Calculated = (calcium content reckoned on fresh weight) × 2·60.

fresh spinach, were found to have an average calcium content of 15·9 % in their bones, whereas the four control animals, which received the basal diet without supplement, had an average calcium content of only 14·5 %. The average growth in weight was also better in the former group, the weight of the male animals being increased by 170 %, whereas in case of the control rats the increase was 110 %. Histological examination of the rib-junctions showed a condition of osteoporosis in all the animals and a slight degree of rickets in certain animals of each group.

If the proportion of calcium in the dry bones at the end of the experiment, compared with that of the control animals, be taken as a measure of the calcium-depositing power of the spinach ration, it must be concluded that so small a dose of fresh spinach as 0·1 g. daily has a definite anti-rachitic effect. But it is a curious fact that, whereas this small dose raised the calcium content in Exp. 1 by 10 to 15 %, an increase in the spinach ration to ten times this amount, to 1·0 g. daily, made no further difference. The figure obtained for animals receiving the larger ration, 16·8 % Ca, was still far below the normal for animals of this age, viz. 20·5 % [see Korenchevsky, 1922]. The body weight was, however, increased in proportion to the spinach ration.

In the subsequent experiments the analyses of the bones were made more complete by extracting the fatty material before making the calcium determinations.

In general it is true to say that the bones of the control animals contained much more fat than those of animals receiving spinach (see Table VI), exceptions occurring only in those control animals which became sick and died before the end of the experiment or were killed after a period of semi-starvation and loss of body weight (*e.g.* rat 131, Table III). The difference in bone calcium between control animals and those receiving spinach therefore appears considerably less if the calculation is made upon the weight of the fat-extracted bones instead of upon the dry weight, see Tables VI and VII. It is probable, therefore, that the apparent improvement in calcification due to the spinach ration in Exps. 1 and 2 would have disappeared had the analyses been made on the fat-extracted bones. In any case this supposition affords the most likely explanation of the fact that increase of the ration failed to cause any corresponding increase in the calcium content of the bones.

Exp. 3. *Early spring spinach grown February–April*, 1925. This experiment included 28 rats from 5 different litters and the doses of spinach varied from 0·1 g. to 2·6 g. daily. Complete details concerning 2 of the 5 litters are set out in Table III and a summary of the whole experiment is given in Table IV. The growth of the animals receiving spinach was much stimulated, the ophthalmia was less or was entirely prevented, and the general condition was much improved in comparison with that of the control animals. But the calcium content of the bones, whether reckoned on the dry, or the extracted dry weight, showed no increase over that of the control animals even in the case of the animals receiving the larger rations. The total variation is from

15·1 % to 15·7 % Ca, reckoned on the dry weight and from 16·6 % to 17·5 % Ca, reckoned on the fat-extracted weight. These values, on the other hand, are definitely lower than those obtained for the animals receiving cod-liver oil or ultra-violet irradiation; in this case the figures range from 17·4 % to 18·4 % Ca, reckoned on the dry weight and from 19·6 % to 21·9 % Ca, reckoned on the fat-extracted dry weight (see Table IV).

Table IV.

Exp. 3. Diet F.R. Early spring spinach. February–April 1925.
Duration of experiment 37–52 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones: % on dry wt.	Average calcium content of bones: % on extracted dry wt.	Ratio A/R
0, Control animals	8	86	15·1	17·1	0·85
Spinach 0·1 g.	6	187	15·4	17·2	0·90
0·5 g.	4	212	15·7	17·5	0·94
(averages) 1·4–2·6 g.	5	248	15·1	16·6	0·82
Cod-liver oil, 1 drop = 0·02 g.	2	247	18·6	20·4	1·23
Irradiated animals:					
0, Control animals	1	150	18·4	21·0	1·32
Spinach 0·5 g.	1	123	17·4	19·6	1·13

The detailed analysis of the fresh bones given in Table III shows a striking constancy among all groups of animals in the organic residue of the bone after the fat had been extracted. The larger variations in bone composition seem to be confined to the water, fat and mineral constituents. These three components consistently amount to 77 % to 80 % of the total weight, leaving 23 % to 20 % for the organic residue. It seems probable that the value of the ratio *mineral ash/organic residue* might after all be the most trustworthy chemical guide to the efficiency of the process of calcification. In the tables A/R is used to signify this ratio.

In Exp. 3 this ratio showed no consistent increase corresponding to the inclusion of spinach in the diet, though the effect of irradiating the animal or of giving cod-liver oil is well marked. The histological investigation of the rib-junctions of the rats confirmed the results of the chemical analysis. The bones of the control rats showed a condition of osteoporosis. Those of the animals receiving spinach had in addition an increased zone of proliferating cartilage and more osteoid tissue, indicating a slight degree of rickets and a less degree of osteoporosis. In litter 140, for example, rat 130, receiving an average of 2·6 g. spinach daily, had a fractured fibula and four fractured ribs. The post-mortem examination and the histology of the rib-junctions showed a slight degree of rickets of which there was no trace in the control animals of the same family. Rat 130 had grown at the normal rate during the 51 days of the experiment, whilst the growth of the control animals was at less than one-half the normal rate.

The spinach used in this experiment was grown in the winter and early spring. The later experiments were therefore made with leaves grown in summer, to see whether the increased amount of ultra-violet light falling on the leaves might improve their anti-rachitic value.

Exps. 4 *and* 5. *Summer-grown spinach, May–October*, 1925. Tests were made with spinach grown from May to October and for comparison with each batch raised in open ground ("open" spinach), a crop was raised simultaneously in the same situation, but covered with a well-ventilated frame glazed with Crookes' "A" glass ("frame" spinach). This glass, which is clear but possesses a slight yellow tint, has the property of absorbing ultra-violet radiations of wave-length shorter than 3780 Å while transmitting 88 % of the visible light. To provide a standard for comparison with the two types of spinach, the "open" spinach fed to certain animals in Exp. 5 was irradiated immediately before consumption for 30 minutes at a distance of 36 cm. from a quartz mercury vapour arc lamp.

Exp. 4 was made with spinach grown from May to July, the period of the year when the sun's illumination lasts longest and is most intense. In 1925, this period included some intervals of very bright weather. The animals received the F.R. diet and the experiment was somewhat complicated by the occurrence of persistent looseness of stools among many of the animals, especially those which were irradiated. Table V gives a summary of observations on 36 rats from 5 litters, the various doses of spinach being distributed as evenly as possible among the animals of the different families.

Table V.

Exp. 4. Diet F.R. Summer spinach. May–July 1925.
Duration of experiment 40–44 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones: % on dry wt.	Average calcium content of bones: % on extracted dry wt.	Ratio A/R
0, Control animals	9	73	13·6	16·9	0·88
"Frame" spinach 0·1 g.	2	178	14·9	17·4	0·94
0·5 g.	3	199	16·1	17·5	0·95
(averages) 1·0–2·5 g.	5	171	15·0	17·0	0·90
"Open" spinach 0·1 g.	3	154	14·8	17·3	0·93
0·5 g.	2	210	15·7	17·7	0·99
(averages) 0·9–2·0 g.	4	182	15·7	17·3	0·93
Cod-liver oil, 1 drop = 0·02 g.	5	172	17·0	18·7	1·08
Irradiated animals:					
0, Control animal	1	47	15·9	17·8	0·97
"Frame" spinach 0·1 g.	1	155	17·6	19·6	1·27
"Open" spinach 0·1 g.	1	93	16·5	18·2	1·03

In this experiment the spinach rations do appear to exert a small but definite influence in increasing the calcification of the bones, and the "open" spinach seems to be more effective than the "frame," whichever criterion is adopted for judging the effect. The calcium content of the bones is raised,

Table VI.

Exp. 5. Diet F. (Ca=0·4 %; P=0·9 %, reckoned on the dry weight.) Late summer spinach. August–October 1925.

Litter	Rat no.	Sex	Dose, daily	Duration of experiment, days	Body weight: Initial g.	Body weight: Increase %	Analysis of bones, calculated on fresh weight, %: H_2O	Fat	Ash	Residue	Ratio A/R	Ca %*: on dry wt.	Ca %*: on extr. dry wt.	Histology of rib-junctions	Ophthalmia	Remarks
260	188	♂	0, Control	43	31	45	47·3	13·7	19·0	20·0	0·95	12·9	17·5	Osteoporosis	Severe	Growth very poor
	196	♂	0, ,,	44	31	58	41·7	13·3	23·8	21·2	1·12	14·7	19·0	,,	Slight	Growth poor
	191	♂	"Open" spinach 0·5 g.	43	33	94	53·0	7·6	19·6	19·8	0·99	15·0	17·9	Osteoporosis and ? very slight rickets	Very slight	Growth poor, P.M. 5 fractured ribs
	192	♂	,, ,, 0·5 g.	43	33	106	48·6	5·9	23·9	21·7	1·10	16·8	19·0	Slight osteoporosis	None	Growth subnormal
	190	♂	,, ,, 1·6 g. (av.)	43	31	116	50·5	3·8	21·7	24·0	0·90	15·8	17·1	Slight osteomalacia	Very slight	,, ,,
	189	♂	,, ,, 2·6 g. (av.)	43	34	150	52·7	4·6	20·9	21·8	0·96	15·9	17·7	,, ,,	None	,, ,,
	193	♂	"Frame" spinach 0·5 g.	43	34	121	48·2	6·7	23·4	21·7	1·07	16·2	18·6	Nearly normal	Doubtful	,, ,,
	195	♀	,, ,, 0·5 g.	43	35	89	46·0	8·0	24·3	21·7	1·12	16·2	19·0	,, ,,	Slight	,, ,,
	194	♀	Irradiated "open" spinach, 0·5 g.	43	31	190	45·7	2·3	29·4	22·6	1·30	19·5	20·3	Normal	None	,, ,,
264	197	♂	0, Control	44	35	54	43·3	16·9	19·8	20·1	0·98	12·6	17·8	Osteoporosis and ? slight osteomalacia	Doubtful	Growth slightly subnormal
	204	♀	0, ,,	43	38	37	44·9	15·8	19·2	20·2	0·95	12·5	17·5	Osteoporosis and ? slight osteomalacia; infraction	,,	Growth very poor
	200	♂	"Open" spinach, 0·5 g.	43	40	55	46·5	14·0	19·1	20·4	0·94	12·9	17·4	Osteoporosis		Growth very poor. P.M. 2 fractured ribs
	198	♂	,, ,, 1·0 g. (av.)	43	37	100	46·8	11·5	20·5	21·1	0·97	13·9	17·7	Osteoporosis and very slight osteomalacia	,,	Growth poor
	202	♂	"Frame" spinach, 0·65 g. (av.)	43	37	81	48·2	9·7	20·7	21·4	0·97	14·4	17·7	Slight osteoporosis	Slight	Growth subnormal
	199	♂	Irradiated "open" spinach, 0·5 g.	43	39	138	43·7	9·5	25·5	21·3	1·20	16·3	19·6	Slight osteoporosis, nearly normal	Doubtful	Growth poor
	205	♀	Irradiated "open" spinach, 1·9 g. (av.)	43	36	200	44·4	5·5	28·4	21·7	1·31	18·3	20·4	,, ,,	None	Growth subnormal
	201	♀	Cod-liver oil, 3 drops	44	36	200	43·5	7·5	27·7	21·3	1·30	17·7	20·4	Normal	Doubtful	Growth subnormal, improving later
	203	♀	,, ,, ,,	43	33	164	45·7	5·1	27·6	21·6	1·28	18·3	20·2	,,	None	,, ,,
261	212	♂	0, Control	44	35	83	43·4	11·5	24·0	21·2	1·13	15·2	19·1	Osteoporosis	Very slight	,, ,,
	208	♂	0, ,,	44	37	59	43·3	11·1	24·5	21·1	1·16	15·6	19·4	,,	Doubtful	Growth poor
	213	♂	"Open" spinach, 0·5 g.	44	36	72	46·3	8·6	23·6	21·5	1·10	15·8	18·9	Less osteoporosis; increased osteoid in shaft	None	Growth very poor
	209	♂	,, ,, 2·0 g. (av.)	44	39	103	45·1	8·6	24·5	21·8	1·08	16·0	19·0	,,	,,	Growth poor
	211	♂	"Frame" spinach, 0·5 g.	44	37	76	46·2	10·5	22·2	21·1	1·05	14·9	18·5	Osteoporosis"	Doubtful	Growth subnormal
	210	♂	,, ,, 1·2 g. (av.)	44	35	94	45·5	7·2	24·1	22·3	1·08	16·0	18·4	Osteoporosis; increased osteoid in shaft	,,	Growth poor
	207	♀	Irradiated "open" spinach, 0·5 g.	44	34	156	44·2	4·7	29·5	21·6	1·36	19·0	20·8	Nearer normal than control	None	Growth subnormal

* Calculated =36% of ash.

whether calculation is made on the dry, or on the extracted dry weight, and the value of the ratio A/R is also definitely increased. In all three cases however the figures are lower than those of corresponding animals which had received ultra-violet irradiation (Table V).

Exp. 5 (Tables VI and VII) was made with spinach grown in late summer, from August to October. The results recall those of Exps. 1 and 2. The addition of "open" or "frame" spinach, whilst stimulating growth and preventing xerophthalmia, caused no improvement in the calcification of the bones. The calcium content, reckoned on the dry weight, was raised relatively to that of the control animals, but the figures calculated on the fat-extracted weight showed no increase and there is none in the A/R ratio.

Table VII.

Exp. 5. Diet F. Late summer spinach. August–October 1925.
Duration of experiment 43–44 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones		
			% on dry wt.	% on extracted dry wt.	Ratio A/R
0, Control animals	6	56	13·9	18·4	1·05
"Frame" spinach 0·5 g.	3	95	15·8	18·7	1·08
(averages) 0·65–1·2 g.	2	88	15·2	18·0	1·02
"Open" spinach 0·5 g.	4	82	15·1	18·3	1·03
(averages) 1·0–2·6 g.	4	117	15·4	17·9	0·96
Irradiated "open" spinach 0·5 g.	3	161	18·3	20·2	1·29
(average) 1·9 g.	1	156	18·3	20·4	1·31
Cod-liver oil, 3 drops = 0·06 g.	2	182	18·0	20·3	1·29

The histological investigation of the ribs of the animals receiving spinach showed on the whole a less degree of osteoporosis than was present in the control animals, and in addition a tendency to slight osteomalacia as evidenced by an increased amount of osteoid tissue in the shaft of the bone. The bone cartilage junction showed no increased tendency to disorganisation as in the rats receiving spinach in Exp. 3. The animals in Exp. 5 were a little heavier at the beginning of the experiment, 31 to 40 g. in weight instead of 20 to 30 g., and the F diet was used with its higher proportion of Ca and P, instead of the F.R. diet. This may account for the different type of the departure from the normal.

The artificially irradiated spinach had a very marked effect upon the calcification of the bones. The average calcium content of the fat-extracted bones was increased from 18·4 % Ca to 20 % Ca and the A/R ratio was increased from 1·06 to 1·31 (Table VII). The histology of the rib-junctions was more nearly normal than in the case of the control animals (see Table VI).

Discussion.

The above experiments confirm the observations made by Hess and Weinstock [1924, 1925] showing that young green wheat or fresh lettuce leaves had no power to prevent rickets in rats, but acquired anti-rachitic properties when irradiated with ultra-violet light from an artificial source. These tests were made with diets deprived of fat-soluble vitamins and at the same time very low in phosphorus, whereas the diets employed in the present work were devoid only of fat-soluble vitamins. It was thought that anti-rachitic properties might be revealed under the latter conditions which were masked under the more severe tests made with diets deficient in phosphorus.

This has proved not to be the case with the exception of Exp. 4, in which spinach grown under the most favourable conditions of natural illumination appeared to possess a slight calcium-depositing effect on the bones of the rats which consumed it.

It is difficult to understand why direct sunlight falling on the green leaves should not confer definite anti-rachitic properties, seeing that irradiation with ultra-violet light from artificial sources does so in so marked a degree. In other cases the biological action of the two types of radiation has been found to be exactly comparable. Rickets in children or in animals is prevented or cured equally by exposure to either. The reaction of animals sensitised with eosin or haematoporphyrin is also similar when exposed to either type of light. The difference found in the case of green leaves might be due

(1) to a difference in the *degree of intensity*, the sun's radiation even under the most favourable circumstances being much feebler in ultra-violet rays of certain wave-lengths, or

(2) to a rapid removal or decomposition of any anti-rachitic substances that might be produced in the growing leaf.

In the present case the spinach leaves were usually cut two or three times a week after sunset, placed in airtight tins over night and then kept in the dark at a low temperature until fed to the animals. This procedure was adopted in order to prevent any exposure of the "frame" spinach to unfiltered sunlight. It was therefore conceivable that any anti-rachitic powers possessed originally might have been lost during the period of storage.

Hess and Weinstock, however, found that irradiated cut lettuce leaves and green wheat seedlings retained their anti-rachitic potency after being stored for 3 days and 2 weeks respectively. It does not therefore seem likely that anti-rachitic substances are formed in the leaf tissue and then rapidly destroyed, although the conditions of a growing leaf attached to the plant might be different in this respect from those of a cut leaf or shoot. It would be interesting to investigate the leaves of summer-grown spinach fed to animals immediately after being gathered in the middle of the day.

It is conceivable that the high vitamin A content of green leaves might prevent the detection of vitamin D, if present in small amount. The rats

receiving spinach grew much larger than the control animals and were better animals in every way. The histology of the rib-junctions showed a greater tendency to rickets, but the bones were equally well, and in Exp. 4 better, calcified, notwithstanding the greater strain imposed by the greater growth. This fact is also brought out by the work of Boas [1926] in which direct measurements of calcium and phosphorus retention were made in animals upon diets deprived of fat-soluble vitamins with and without additions of the fresh leaves of winter spinach. It is possible that a marked disproportion between the respective amounts of vitamins A and D provided in the additional ration might limit the effectiveness of the latter if present in very small quantity. The recent work of Mellanby [1925], showing the effect of certain cereals in increasing the severity of rickets in dogs when added to a rickets-producing diet, may have an analogous explanation. The potency of the different cereals will be found to be in proportion to their content of vitamin A, wheat germ, oatmeal and whole wheat flour being more potent than rice or white wheat flour. At the same time definite anti-rachitic properties were demonstrated in the unsaponifiable fractions isolated from the fat contained in both oatmeal and wheat germ.

The failure to demonstrate any appreciable anti-rachitic potency in green leaves exposed to natural sunlight raises the question of the origin of the anti-rachitic vitamin in nature. It may well be that vitamin D is present but that the amount is small and can only be detected in cases in which large quantities of leaves are consumed, as in the experiments of Shipley, Kinney and McCollum [1924] with dried alfalfa. Herbivorous animals in the natural state are certainly protected from rickets, but they take a diet composed almost exclusively of fresh green food and are at the same time themselves exposed to the sun's direct radiation. The origin of the rich stores of anti-rachitic vitamin in the liver of the cod and other fishes is a more difficult problem. For these animals spend most of their life at a depth below the surface of the water at which they must be exposed only to a very small fraction of the total ultra-violet radiation of the sun. Their food has been traced ultimately to green algae which are intermittently exposed to direct sunlight, but these, if at all similar to the green leaves of land plants, may be assumed to be but poorly endowed with the anti-rachitic substance. It would be worth while to make a more complete study of some marine green diatoms, such as Nitzschia, which has been shown by Jameson, Drummond and Coward [1922] to be rich in vitamin A, but whose anti-rachitic value has not yet been investigated.

Summary.

1. Fresh leaves of spinach are a rich source of vitamin A, a small daily ration (0·1 g. and upwards) encouraging growth and lessening or preventing xerophthalmia in young rats on diets devoid of fat-soluble vitamins.

2. Spinach grown in the open in winter, spring or autumn possesses no

anti-rachitic properties that can be demonstrated by the methods employed. Spinach grown in midsummer has a slight but appreciable anti-rachitic value.

3. Spinach leaves when irradiated with ultra-violet rays from a mercury vapour quartz lamp become powerfully anti-rachitic.

4. There is no obvious explanation for this discrepancy between the effect of direct sunlight and of artificial ultra-violet radiation. The difference may be one of degree or there may be a rapid removal or destruction of newly formed anti-rachitic substance in the leaves while these are attached to the living plant. It is also possible that the failure to demonstrate anti-rachitic properties in naturally illuminated spinach may be due partly to their disproportionate content of vitamin A.

Our thanks are due to Miss Mary Tazelaar and to Miss Joan Leigh-Clare for help in feeding and care of the animals, and to Miss Sheila Rutherford for preparation of the histological specimens.

Note on the criteria adopted for the diagnosis of rickets, based upon histological examination. By V. KORENCHEVSKY.

THE principal pathological change found in the bones of the rats described above was one of osteoporosis. In addition to this, the rib-junctions of some rats showed a slight or very slight degree of rickets. This consisted of an increased amount of osteoid tissue in relation to the thickness of the trabeculae or of the shaft walls. The proliferating cartilage was also increased in some cases to a slight or moderate degree and there was also a partial deficiency in the calcification of the zone of provisional calcification.

In some rats of Exp. 5 there was an increased amount of osteoid tissue in the shaft of the bones without any increase in the proliferating cartilage or deficiency of calcium in the zone of provisional calcification. These cases were diagnosed as osteomalacia.

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XXIII. THE ANTI-RACHITIC VALUE OF WINTER SPINACH[1].

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1. The Influence of Fresh Green Winter Spinach upon the Retention of Calcium and Phosphorus by Young Rats.

An account of previous observations upon the content of the anti-rachitic factor (vitamin D) in fresh green leaves is given in the preceding paper [Chick and Roscoe, 1926] and therefore only those investigations will be referred to here in which the Ca and P retention of animals fed upon green food was studied.

Hart, Steenbock and Hoppert [1921], in a study of the Ca metabolism of a goat, found that the retention of this substance was increased by the addition of fresh green oats to an otherwise dry diet; raw cabbage did not however seem to have any such effect. Hart, Steenbock, Hoppert and Humphrey [1922] working on milking cows converted a negative Ca balance into a positive one by adding dry alfalfa hay to the diet, whilst the substitution of this by fresh green alfalfa further increased the storage of Ca. In two cases out of three the storage of P was also increased. Hart, Steenbock, Hoppert, Bethke and Humphrey [1922] changed negative Ca and P balances in milking cows into positive ones by replacing the dry timothy hay in the diet by green alfalfa hay. Gaessler and McCandish [1923] found positive Ca balances in cows fed upon a diet with a low fat content but containing large amounts of fresh alfalfa hay. When this was replaced by alfalfa hay which was $1\frac{1}{2}$ years old a negative balance appeared.

The results of these investigations agree in demonstrating the influence of green plants in raising the Ca retention of herbivorous animals fed upon them, and in showing that the fresh green plants are superior in this respect to the dried material.

The present paper records an investigation of the effect of the fresh green leaves of winter spinach upon the Ca and P metabolism of the young rat. For this purpose the retention of Ca and P was compared in groups of young rats fed in the following three different ways:

[1] The first experiment in this paper formed part of a thesis approved for the degree of Doctor of Philosophy in the University of London.

(1) upon a basal control diet deprived of fat-soluble vitamins,
(2) upon the same diet supplemented by a daily ration of fresh green spinach leaves, and
(3) upon the basal diet supplemented by a daily ration of cod-liver oil.

The metabolic experiments were of two types. In the first type a litter of rats was divided into three groups, each of which received one of the above diets, the retention of Ca and P in each group being compared with that in the others. In the second type of experiment the same rats received first the basal diet alone for some weeks, and subsequently the basal diet supplemented by spinach.

Experimental Methods.

The method for estimating Ca and P retention already described [Boas, 1924] was employed. Certain refinements were introduced, the most important being the separate collection of faeces, urine and uneaten food. The glass separators designed by Gross and Connell [1923] were used and proved satisfactory.

Two different basal diets were used.

Basal Diet 1 had the following composition:

Inactivated caseinogen	120 g.
Wheaten starch	342 ,,
Yeast extract	36 ,,
Hardened sesame oil	90 ,,
Salt mixture [see Boas, 1924]	10·5 ,,
Ferric citrate	0·5 ,,
Orange juice	36 c.c.
Distilled water	280 ,,

Sufficient $CaCO_3$ and K_2HPO_4 were added to produce a diet with a final Ca content of 0·54–0·57 % and a P content of 0·54–0·60 % reckoned on the wet weight, 0·95 % Ca and 0·96 % P reckoned on the dry weight.

Basal Diet 2 had the following composition:

Inactivated caseinogen	100 g.
Wheaten starch	250 ,,
Yeast extract	25 ,,
Hardened cotton seed oil	75 ,,
Ferric citrate	0·4 ,,
Calcium carbonate	3·0 ,,
Potassium monohydrogen phosphate ...	3·8 ,,
Salt mixture [see Boas, 1924]	9·0 ,,
Lemon juice	25 cc.
Distilled water	300 ,,

In this case the salts containing Ca and P were mixed with the dry constituents of the diet. Two samples of each batch of the diet after the water was added were taken for the determination of the content of Ca and P.

This averaged 0·24 % Ca and 0·26 % P reckoned on the wet weight, 0·44 % Ca and 0·48 % P reckoned on the dry weight of the diet. This diet had therefore a considerably lower Ca and P content than the first basal diet.

A normal mixed diet for a rat, consisting of milk, brown bread and cabbage, contains about 0·23 % Ca and 0·32 % P reckoned on the dry weight, and it was therefore thought that the first basal diet contained an amount of these substances far in excess of the normal requirement though it was similar to the diets used by other workers [see Goldblatt, 1923, p. 301].

The leaves of prickly seeded winter spinach (*Spinacea oleracea*) were used. After being carefully washed and dried the stalks and large ribs were removed. All leaves which were at all discoloured were rejected. At the beginning of each week the weekly ration was weighed out to the nearest gram into a stoppered bottle and placed in the refrigerating room, a representative sample being taken at the same time for the estimation of Ca and P content. Each rat received its ration separately, but if not consumed within reasonable time the residue was mixed with the basal ration. The successive batches of spinach had a Ca content ranging from 0·12 % to 0·21 % and a P content of from 0·0 % to 0·13 % of the fresh leaves. The water content averaged 90 %.

Experiments and Results.

Three metabolism experiments each lasting for 8 to 11 weeks were carried out on young rats.

Exp. 1 (*February to May*, 1924). Six female rats from one litter were placed in couples in three metabolism cages when they were 22 days old and had an average body weight of 44 g. In the first group (Group A) the rats received basal diet 1. In the second group (Group C) each rat received 3 drops of cod-liver oil daily in addition to the basal diet, whilst the rats comprising the third group (Group S) were given a daily ration of fresh spinach starting at 1 g. per rat, and increasing to 5 g. before the end of the first fortnight. The ration was kept at this level during the rest of the experiment. The first fortnight was used as a preliminary period during which no determinations were made, and followed by three periods, each consisting of two weeks, during which the excreta were collected and the retention of Ca and P was estimated.

Results of Exp. 1. Fig. 1 shows the mean curve of increase in body weight per rat of the three groups during the entire eight weeks of the experiment. The rats receiving cod-liver oil gained on an average 69 g. per rat, those receiving spinach (Group S) 79 g., whilst the controls in Group A gained no more than 45 g. Tables II and III contain the average intake, output and retention per rat of Ca and of P respectively during the three periods of the experiment. In Fig. 2 the average successive total increments of Ca and P per rat are shown plotted against the time. Table III contains the data obtained by analysis of the leg bones of the rats.

Table I. *The calcium metabolism (average per rat) of the six female rats in Exp.* 1.

GROUP C. Rats receiving basal diet and a daily ration of 3 drops of cod-liver oil per rat.

Period	Rise in body wt. g.	Intake g.	Output: Faeces g.	Output: Urine g.	Output: Total g.	Retention g.
Preliminary (2 weeks)	47– 71					
1. (2 weeks)	71– 97	1·0672	0·7565	0·0239	0·7804	0·2868
2. (,,)	97–122	1·1614	0·8600	0·0238	0·8838	0·2776
3. (,,)	122–140	1·0676	0·8728	0·0157	0·8885	0·1791
Total (6 weeks)	71–140	3·2962	2·4893	0·0634	2·5527	0·7435

GROUP S. Rats receiving basal diet and a daily ration of 5 g. of fresh green spinach leaves per rat.

Preliminary (2 weeks)	45– 73					
1. (2 weeks)	73–100	1·1950	0·8608	0·0195	0·8803	0·3147
2. (,,)	100–132	1·4175	1·1340	0·0223	1·1563	0·2612
3. (,,)	132–152	1·2561	1·0835	0·0161	1·0996	0·1565
Total (6 weeks)	73–152	3·8686	3·0783	0·0579	3·1362	0·7324

GROUP A. Rats receiving basal diet only.

Preliminary (2 weeks)	47– 60					
1. (2 weeks)	60– 69	0·7976	0·6573	0·0085	0·6658	0·1318
2. (,,)	69– 89	0·8549	0·7037	0·0103	0·7140	0·1409
3. (,,)	89–105	0·8680	0·7062	0·0118	0·7180	0·1500
Total (6 weeks)	60–105	2·5205	2·0672	0·0306	2·0978	0·4227

Table II. *The phosphorus metabolism (average per rat) of the six female rats in Exp.* 1.

GROUP C. Rats receiving basal diet and a daily ration of 3 drops of cod-liver oil per rat.

Period	Rise in body wt. g.	Intake g.	Output: Faeces g.	Output: Urine g.	Output: Total g.	Retention g.
Preliminary (2 weeks)	47– 71					
1. (2 weeks)	71– 97	1·1437	0·3661	0·5676	0·9337	0·2100
2. (,,)	97–122	1·2608	0·4201	0·6913	1·1114	0·1494
3. (,,)	122–140	1·1402	0·4488	0·5760	1·0248	0·1154
Total (6 weeks)	71–140	3·5447	1·2350	1·8349	3·0699	0·4748

GROUP S. Rats receiving basal diet and a daily ration of 5 g. of fresh green spinach leaves per rat.

Preliminary (2 weeks)	47– 73					
1. (2 weeks)	73–100	1·2710	0·3280	0·7263	1·0543	0·2167
2. (,,)	100–132	1·4591	0·4519	0·8415	1·2934	0·1657
3. (,,)	132–152	1·2999	0·4102	0·7693	1·1795	0·1204
Total (6 weeks)	73–152	4·0300	1·1901	2·3371	3·5272	0·5028

GROUP A. Rats receiving basal diet only.

Preliminary (2 weeks)	47– 60					
1. (2 weeks)	60– 69	0·8379	0·3419	0·3799	0·7218	0·1161
2. (,,)	69– 89	0·9164	0·3660	0·4461	0·8121	0·1043
3. (,,)	89–105	0·9457	0·3703	0·4861	0·8564	0·0893
Total (6 weeks)	60–105	2·7000	1·0782	1·3121	2·3903	0·3097

Table III. *Analyses of leg bones (femora, tibiae and fibulae) dried at* 110° *for* 24 *hours of the six female rats in Exp.* 1.

Rat No.	Diet	Body wt. g.	Dry wt. of leg bones g.	Dry wt. of bones, % of body wt. (average of 2 rats)	Ca content of bones, % of dry wt.	
61	Cod-liver oil	149	0·9345	0·66	20·44	19·78
62	,,	130	0·8957		19·12	
65	Spinach	156	0·8339	0·56	18·74	18·66
66	,,	148	0·8701		18·57	
63	Basal diet only	99	0·6609	0·74	17·89	17·52
64	,,	105	0·7764		17·14	

At first sight it would appear from these tables that the addition of spinach to the diet has promoted the retention of Ca to as great a degree as has the ration of cod-liver oil. When however the data are carefully examined this proves not to be the case.

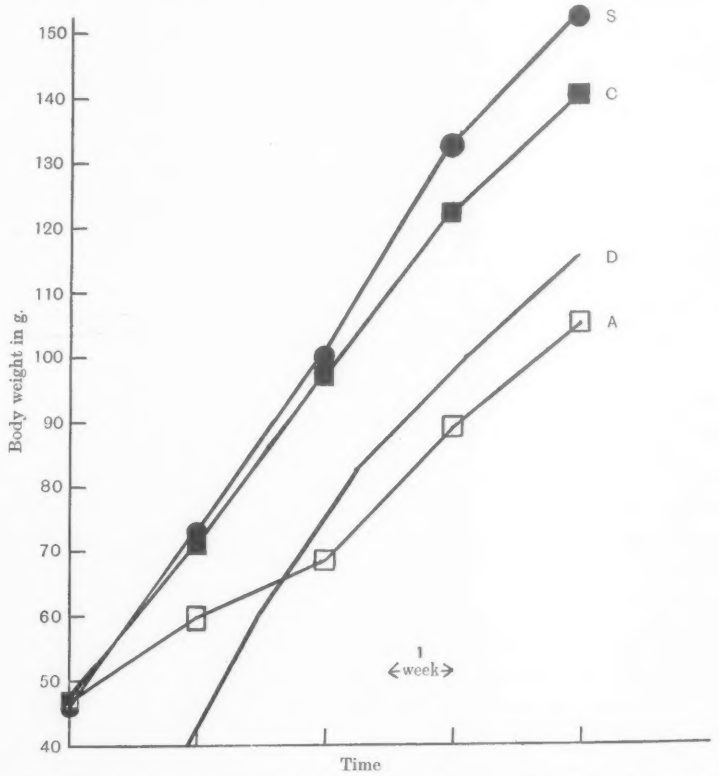

Fig. 1. Curves showing the increase in body weight of the rats in Exp. 1, receiving (A) basal diet only, (C) basal diet and cod-liver oil, and (S) basal diet and spinach. Each curve represents average figures for two female rats from the same litter. D is the Wistar Institute's curve of normal growth for female rats.

The rats receiving cod-liver oil had a final body weight 33 % heavier than that of the controls and their bodies contained more fat. Their leg bones (see Table III) were 25 % heavier and had a Ca content which was 12 % higher when reckoned on the dry weight. Chick and Roscoe [1926] find that the bones of rats deprived of fat-soluble vitamins contain more fat than do those of rats receiving cod-liver oil or spinach. Therefore it may be assumed that could the content of Ca in the fat-free bone be calculated, the difference in Ca content would be less.

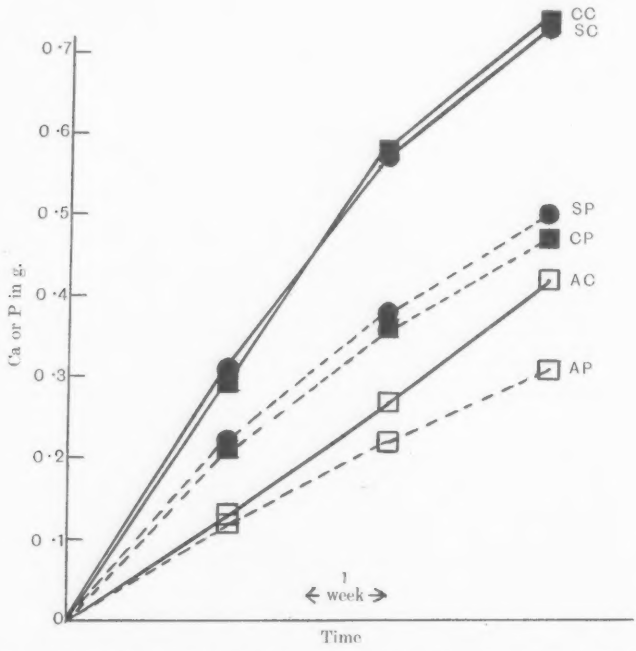

Fig. 2. Curves of Ca retention by the rats in Exp. 1 receiving basal diet only (AC), basal diet and cod-liver oil (CC) and basal diet and spinach (SC). Curves AP, CP and SP show the respective P retentions of the three groups.

The amount of Ca retained for every 10 g. increase in body weight (see Table IV) was 15 % higher in Group C than in Group A. Figures given by Donaldson [1924] show that at this time of life in a normal rat the increase in weight of the skeleton is very nearly proportional to that of the whole body. That this was so in the rats receiving cod-liver oil is shown by the fact that during the successive periods of the experiment the retention of Ca per 10 g. increase in body weight remained level (0·110, 0·112, 0·100). The above figures show that the favourable influence of cod-liver oil upon growth and Ca retention is clearly demonstrated in this experiment.

Table IV. *The amounts of Ca and P retained per* 10 *g. increase in body weight by the rats in Exp.* 1.

Group	Addition to basal diet	1st fortnight Ca g.	1st fortnight P g.	2nd fortnight Ca g.	2nd fortnight P g.	3rd fortnight Ca g.	3rd fortnight P g.	Total period Ca g.	Total period P g.
C	Cod-liver oil	0·110	0·075	0·112	0·060	0·100	0·064	0·108	0·068
S	Spinach	0·116	0·080	0·082	0·075	0·074	0·040	0·092	0·063
A	None	0·146	0·130	0·070	0·052	0·094	0·057	0·094	0·069

The rats receiving spinach (Group S) also grew well and were 44 % heavier than the controls (Group A) and 11 % heavier than the rats receiving cod-liver oil (Group C) at the end of the experiment. The weight of the skeleton was not however increased in proportion, the leg bones being only 18 % heavier than those of Group A and 7 % lighter than those of Group C. The content of Ca in the leg bones of Group S reckoned on the dry weight was 6 % more than in Group A; for the reasons given above it is probable that the percentage of Ca in the fat-free bone would show a smaller difference. Over the entire six weeks of the experiment the average amount of Ca retained per unit increase in body weight was the same in Groups A and S (0·094 and 0·092 g. respectively). In Group S there was a steady decrease in this ratio during the course of the experiment, indicating that the calcification of the skeleton was *declining* relatively to the increase in weight of the body. These results lead one to the conclusion that the winter spinach improved the growth both of the skeleton and of the body as a whole and produced rats stronger and more healthy than were the controls, but there is no evidence that the *degree of calcification of the bones* was improved. It seems probable from Chick's results that had summer spinach been employed the level of Ca storage might have been slightly improved.

Exps. 2 *and* 3 (*October* 1924 *to January* 1925). In each of these experiments four female rats from one litter were placed in pairs in metabolism cages, the two cages being treated as one unit for the purpose of collection and analysis of excreta. The rats received basal diet 2 without any addition for seven weeks. This was Period 1 during the first fortnight of which no analyses were made. During Period 2 which lasted for four weeks a daily ration of spinach was given in addition to the basal diet. The rats received as much spinach as they could be induced to eat—an average of 3·5 g. per day per rat. It was intended to follow Period 2 by a third period during which the spinach would be replaced by a ration of cod-liver oil but this proved impracticable. Such prolonged diarrhoea resulted from the administration of the oil that it was impossible to collect the excreta with a sufficient degree of accuracy.

The results of these experiments confirm those of the first experiment but are not as clearly defined. The stock from which the rats were drawn had been receiving a certain amount of egg-yolk in addition to the usual stock diet which is relatively deficient in the fat-soluble vitamin D. When

the experiments were started it was not realised that the administration of the egg-yolk to the parent animals would result in the young rats possessing such rich stores of fat-soluble vitamins as subsequently proved to be the case. The growth curves given in Fig. 3 show that in Period 1 when the rats were

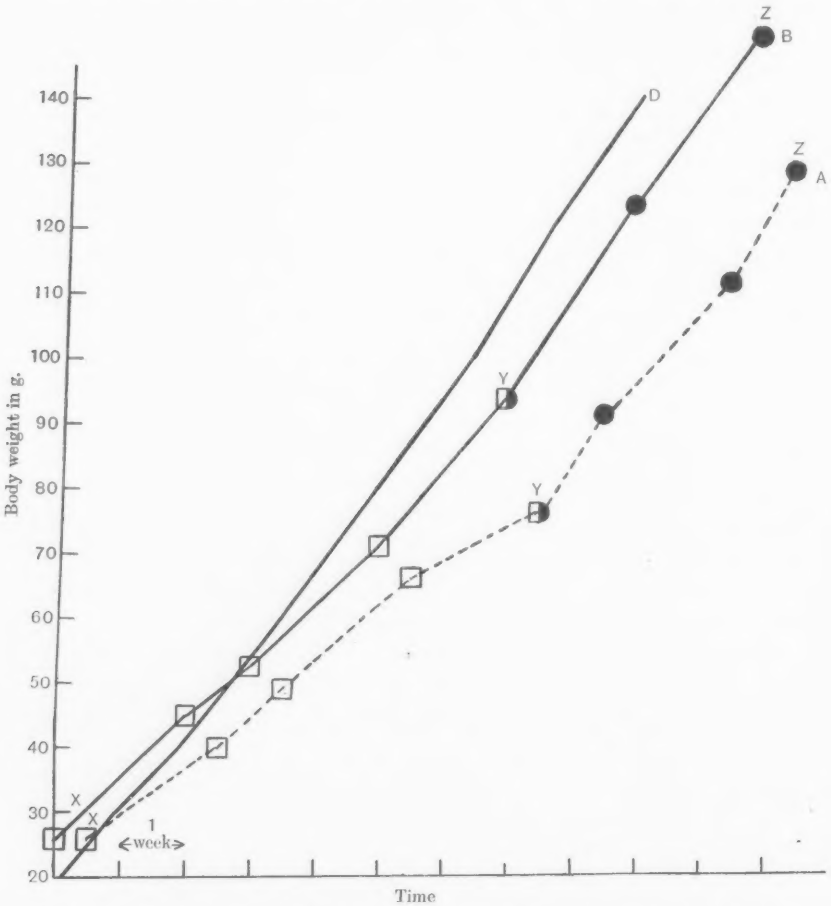

Fig. 3. Curves showing increase in body weight of the rats in Exps. 2 (Curve A) and 3 (Curve B). Each represents average figures for four female rats from one litter. From X to Y the rats received basal diet alone; from Y to Z a daily ration of fresh spinach was given. Curve D is the Wistar Institute's curve of normal growth for a female rat.

receiving basal diet alone, a rate of growth not far below that of a rat fed upon a complete diet was maintained throughout the seven weeks. In both cases the addition of spinach to the diet in Period 2 distinctly stimulated the rate of growth.

Table V. *The average figures for calcium and phosphorus metabolism per rat of the four female rats in Exp.* 2.

During Period 1 basal diet only was given; during Period 2 a daily ration of from 3 to 4 g. of fresh green spinach leaves was ingested by each rat.

		Intake		Output: Faeces		Output: Urine		Output: Total		Retention		Rise in body wt.	Ca retention per 10 g. rise in body wt.
Period	Weeks of Exp.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	g.	g.
1. Basal diet only	1 and 2											26– 40	
	3	0·1556	0·1701	0·0608	0·0354	0·0043	0·0647	0·0651	0·1001	0·0904	0·0700	40– 48	
	4 and 5	0·3786	0·4032	0·2518	0·1480	0·0132*	—	0·2650	—	0·1136	—	48– 66	0·079
	6 and 7	0·3614	0·4012	0·2678	0·1676	0·0108	0·1518	0·2786	0·3194	0·0828	0·0818	66– 76	
2. Basal diet + 3·5 g. of fresh spinach per rat per day	8											76– 91	
	9 and 10	0·5332	0·5594	0·4020	0·1890	0·0154	0·2776	0·4174	0·4666	0·1158	0·0668	91–116	0·038
	11	0·2966	0·3148	0·2618	0·1154	0·0080	0·1689	0·2698	0·2843	0·0278	0·0610	116–128	

* Calculated on the assumption that 95 % of the total Ca excreted was in the faeces, 95 % being the mean of the figures obtained in the preceding and following periods (94 and 96 % respectively).

Table VI. *The average figures for calcium and phosphorus metabolism per rat of the four female rats in Exp.* 3.

During Period 1 basal diet only was given; during Period 2 a daily ration of from 3 to 4 g. of fresh green spinach leaves was ingested by each rat.

		Intake		Output: Faeces		Output: Urine		Output: Total		Retention		Rise in body wt.	Ca retention per 10 g. rise in body wt.
Period	Weeks of Exp.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	Ca g.	P g.	g.	g.
1. Basal diet only	1 and 2											27– 45	
	3	0·1413	0·1497	0·0830	0·0393	0·0032	0·0664	0·0862	0·1057	0·0551	0·0446	45– 53	
	4 and 5	0·3678	0·3938	0·2294	0·1200	0·0102	0·1916	0·2396	0·3016	0·1282	0·0922	53– 71	0·071
	6 and 7	0·4624	0·5112	0·2802	0·1676	0·0150	0·2208	0·2952	0·3884	0·1672	0·1228	71– 94	
2. Basal diet + 3·5 g. of fresh spinach per rat per day	8 and 9	0·6108	0·6420	0·4396	0·2102	0·0158	0·3066	0·4454	0·5168	0·1654	0·1252	94–123	
	10 and 11	0·6194	0·6642	0·4920	0·2154	0·0232	—	0·5152	—	0·1042	—	123–139	0·059

Table V contains figures showing the average Ca and P intake, excretion and retention per rat in Exp. 2, and Table VI gives the same figures for Exp. 3. In Exp. 2 the result of the spinach ration was to raise the absolute amount of Ca retained, but the level of retention in proportion to the increase in weight was lowered from an average of 0·079 g. per 10 g. increase in body weight during Period 1 to an average of only 0·038 g. in Period 2. This shows that the stimulus to growth was not accompanied by a comparable stimulus to the process of calcification.

In Exp. 3 for some reason which it is difficult to see, the retention of Ca and P rose week by week instead of decreasing as is usually the case [see Boas, 1924]. During the first fortnight of Period 2 it remained at the same level and fell during the last fortnight. Here again, as in Exp. 2, in relation to the growth made, the level of Ca retention fell as the result of adding spinach to the diet. The Ca retention per 10 g. increase in body weight fell from 0·071 in Period 1 to 0·059 g. in Period 2.

Exps. 2 and 3 thus provide additional evidence that the inclusion of winter spinach in the diet does not raise the level of Ca retention.

2. The Influence of the Diet upon the Distribution of the Calcium and Phosphorus Excretion between the Urine and the Faeces.

There has been recently a tendency among certain investigators to regard the alimentary canal as the site of the lesion in rickets, and to attribute the disease at any rate in part to a faulty absorption of Ca and P. In consequence more Ca and P would appear in the faeces at the expense of that formerly excreted in the urine, the result of which would be to lower the concentration of these substances in the blood.

Schabad [1909] pointed out that in rickets there was more Ca and P in the faeces and less in the urine than normally. Bertram [1878] stated that in rickets the Ca in the urine was very low. Bogert and Traill [1922] and Givens [1918, 2] were able to divert Ca excretion from faeces to urine by the addition of fat-soluble vitamins to the diet in the form of butter-fat or fresh milk. Orr, Holt, Wilkins and Boone [1923] found that in three cases of rickets in infants the result of ultra-violet irradiation was to improve the retention of Ca and P and to decrease the amounts of these substances excreted in the faeces. In all three cases the amount of P in the urine rose and in two of the cases the amount of Ca also rose.

The administration of acid or of acid-forming diets has been shown to increase the Ca and P in the urine at the expense of that in the faeces by Weiske [1872], Schetelig [1880], Rüdel [1893], Givens [1917, 1918, 1], Kishi [1922] and Bogert and Kirkpatrick [1922], whilst the opposite effect has been shown to occur, when alkalies or base-forming diets are given, by Weiske [1872], Gerhardt and Schelesinger [1899], Wuertz [1913] and Bogert and Kirkpatrick [1922].

Zucker, Johnson and Barnett [1922] showed that the acid-base ratio of the diet could be an important factor in determining whether rickets would or would not develop in rats. The addition of 20 % ammonium chloride to a rickets-producing diet prevented the development of the disease, whilst sodium carbonate changed a non-rickets-producing diet into one which gave marked rickets. It was therefore suggested that in rickets the faulty absorption of Ca and P was due to insufficient acidity of the intestinal tract. Zucker and Matzner [1924] showed that the p_H of a suspension of faeces was about 7·6 in the case of rats receiving their No. 84 rickets-producing diet and that the result of administering the active principle of cod-liver oil, or of irradiation with the mercury vapour lamp was to lower the p_H of the faeces suspension to 5·7–6·4. When the active principle of cod-liver oil was injected subcutaneously no change in the acidity of the faeces took place. Soames [1924] has however shown that the intraperitoneal injection of cod-liver oil confers partial protection against a rickets-producing diet.

Jones, James and Smith [1924] have reported the production of rickets in puppies by the use of diets adequate in all respects but possessing a high potential alkalinity; they were however unable to obtain similar results with

rats. Jones [1924] described three cases of rickets in children in which HCl therapy was used with satisfactory results. Unfortunately in only one case was there no other change in the diet. Stehle [1917] claimed to have increased the storage of Ca in a dog by the administration of HCl, but Givens [1918, 1] and Givens and Mendel [1917] were unable to confirm this, whilst Bogert and Kirkpatrick [1922] concluded that the result of acid-forming diets was to decrease rather than increase the retention of Ca.

There seems therefore to be some evidence for the opinion that in rickets there may be an abnormal alkalinity of the alimentary canal. This would give rise to the formation of insoluble basic salts rather than the more soluble acid salts, and hence to a decreased absorption of Ca and P. The administration of cod-liver oil or irradiation by the mercury vapour lamp are assumed to aid absorption by restoring the normal reaction of the gut. That this is the only action of the anti-rachitic factor and that acid therapy would be as effective as cod-liver oil in the prevention and cure of rickets is difficult to believe, and the experiments of Givens and Mendel, of Bogert and Kirkpatrick and of Soames already referred to are against this view.

Experimental Results.

In the metabolism experiments described above the partition of the Ca and P excretion between the urine and faeces was observed and the data obtained are collected in Tables VII and VIII. Throughout all three experiments 95–99 % of the total Ca excreted was found in the faeces. Neither the addition to the diet of cod-liver oil nor of spinach exerted any appreciable influence upon this partition. It appears therefore that in rats only a very small amount of the Ca is passed in the urine.

Table VII. *The percentage of the total calcium and phosphorus excretion found in the faeces of the rats in Exp.* 1.

Period No.	Group C Basal diet and cod-liver oil		Group S Basal diet and spinach		Group A Basal diet only	
	Ca %	P %	Ca %	P %	Ca %	P %
1	97	39	98	31	99	48
2	97	38	98	35	99	45
3	98	44	99	35	99	43
Mean	97	40	98	34	99	45

Table VIII. *The percentage of the total calcium and phosphorus excretion found in the faeces of the rats in Exps.* 2 *and* 3.

Period	Exp. 2		Exp. 3	
	Ca %	P %	Ca %	P %
1. Rats receiving basal diet only	95	46	96	40
2. Rats receiving basal diet and 3–4 g. of spinach	97	41	96	40

In Exp. 1 the rats in Group A receiving basal diet alone excreted on an average 45 % of the total P excretion in the faeces. The result of adding

cod-liver oil to the ration was to lower this figure to 40 %, whilst in the rats receiving spinach an even smaller proportion of the P excretion was found in the faeces—an average of 34 %.

In Exp. 2 during Period 1 when basal diet alone was given the average percentage of the total P excretion found in the faeces was 47. This was decreased to 41 during the four weeks of spinach ingestion.

The results of Exp. 3 are not of interest. So rich was the store of anti-rachitic factor induced in the rats by the administration of egg-yolk to their parents that seven weeks' deprivation of fat-soluble vitamins proved insufficient to cause any diversion of P from urine to faeces. During both periods of the experiment the proportion of the total P excretion found in the faeces averaged 40 %, the same figure as that obtained in the rats receiving cod-liver oil in Exp. 1.

It is curious that spinach, in spite of its alkaline ash and of the fact that it has been shown to contain little anti-rachitic factor, should yet cause a diversion of P from faeces to urine. There is very little evidence bearing on this point in the literature of the subject. Hart, Steenbock, Hoppert and Humphrey [1922] substituted fresh green alfalfa for dry alfalfa hay as the diet of three milking cows. The storage of Ca and P was increased but the amount of Ca and P in the urine fell and that in the faeces rose. There was however a marked decline in the yield of milk which they attribute to the hot weather and to the presence of flies, so that the animals seem to have suffered from some metabolic disturbance. In a second experiment upon milking cows carried out by the same observers [Hart, Steenbock, Hoppert, Bethke and Humphrey, 1922] timothy hay was changed to green alfalfa hay, and in this case there was no variation in the milk yield. In all three cows the amount of P in the urine rose whilst that in the faeces fell.

It is perhaps dangerous to draw definite conclusions from the results of three metabolism experiments in which only 14 rats were involved but the results obtained offer some evidence against the theory that the anti-rachitic factor promotes calcification by lowering the p_H of the intestine. Two substances, cod-liver oil and spinach, have both been shown to favour the absorption of P as indicated by a relative decrease in the amount of P excreted in the faeces. Cod-liver oil is a rich source of vitamin D but spinach has been shown to contain only negligible quantities of this factor. It follows therefore that although a substance may be able to divert P from faeces to urine there is no ground for assuming that it necessarily possesses anti-rachitic power.

Summary.

1. Direct metabolic experiments have been carried out on young rats in order to compare the influence upon the retention of Ca and P of daily additions to diets deficient in all fat-soluble vitamins of cod-liver oil and of fresh green winter spinach leaves.

2. The addition of cod-liver oil to the diet resulted in an improvement in the general health and an increase in the rate of growth of both the skeleton and the whole body. The degree of calcification of the skeleton was also improved, for there was a rise in the amount of Ca retained per unit increase in body weight.

3. The fresh leaves of winter-grown spinach added to the experimental diet caused an even greater improvement in the well-being of the rats and in the rate of growth. The weight of the skeleton was not however proportionally increased.

4. The conclusion is drawn that winter spinach contains an amount of vitamin D which is negligible compared with its content of vitamin A.

5. 95–98 % of the total Ca excretion of the rat is found in the faeces, and this partition is independent of the amount of fat-soluble vitamins in the diet.

6. Both cod-liver oil and spinach, when added to a diet deficient in fat-soluble vitamins, cause an increase in the amount of P excreted in the urine at the expense of that excreted in the faeces.

I wish to record my thanks to Prof. C. J. Martin and Dr H. Chick for their unfailing help and advice throughout the course of this investigation.

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XXIV. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. IV.

A THEORY OF THE MECHANISM OF OXIDATIONS AND REDUCTIONS *IN VIVO*.

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An attempt will be made, in this communication[1], to interpret in a consistent manner the mechanism of oxidations and reductions *in vivo*. The scheme which will be advanced has formed the basis of the writer's experimental work during the last year and has been developed solely from results with "resting," or non-proliferating, bacteria. No result contradictory to that anticipated by the scheme has, so far, been obtained and what predictions have been made (*e.g.* with formamide, pyrotartaric acid, etc.) have been verified. The scheme, in a certain sense, embraces both Wieland's and Bach's views.

The need for a logical interpretation of the mechanism of oxidation is apparent when the diversity of the material which forms the effective substrate in animal or bacterial oxidations is examined. No very obvious chemical connection appears to exist between those molecules capable of performing the functions of a hydrogen donator in presence of bacteria or muscle tissue (as for instance lactic acid, formic acid, succinic acid) and it seems necessary, in order to comprehend the activity of these molecules, to resort to a hypothesis which claims that for every such molecule there is, related to it, some specific enzyme. The enzyme, on Wieland's views, "activates" the hydrogen of its substrate which then becomes susceptible to oxidation.

Now 103 substances have been examined as possible donators or acceptors of hydrogen (using the methylene blue technique) in the presence of bacteria (*B. coli*), and of these 56 are activated. That is, of the 56 substances some reduce methylene blue, whilst others oxidise leucomethylene blue under con-

[1] The views expressed in this communication are contained in a dissertation which was laid before the authorities of Trinity College, Cambridge, in August, 1924.

ditions (p_H 7·4 and 45°) when they are apparently quite inactive in the absence of the organism. It would follow that we have to imagine that the cell of *B. coli* contains at least 56 specific "hydrogen transportases" (to use Thunberg's terminology), and, *prima facie*, the actual existence, in one cell, of such a large number of specific enzymes dealing with but one type of phenomenon seems very doubtful; moreover, the fact that no success has attended efforts to separate even one—or a group—of these enzymes makes the conclusion all the more improbable.

Granting however, for the time being, that such enzymes may exist, the more important problem of the mechanism of activation by the enzyme awaits consideration. Wieland's theory gives us no information on the actual mechanism of activation and it is, it seems to me, essentially ignorance of this mechanism, rather than the postulation of numerous specific enzymes, which prevents our anticipating, with any measure of certainty, the susceptibility to oxidation of any given molecule. Considerable experience of oxidations in the animal body enables us to state whether a certain molecule is likely to be oxidised *in vivo*, and, with Knoop's β-oxidation rule, to foretell how the molecule may suffer oxidation. But more than this, with no picture of the chemical mechanism of the event, it is impossible to say.

The problem of determining the mechanism of oxidation resolves itself into three separate problems: (1) determination of the site of activation of a substrate molecule; (2) determination of the mechanism of activation of this molecule; (3) determination of the process of oxidation after activation has occurred. These will be examined but it will be useful first to consider one or two difficulties in connection with the specific enzyme hypothesis.

From our knowledge of the behaviour of succinic acid *in vitro* it appears most unlikely that succinic acid (normal or unactivated) will donate hydrogen to a protoplasmic constituent and that the latter will then reduce methylene blue, that is to say, that a protoplasmic constituent will act as a "hydrogen carrier" between succinic acid (unactivated) and methylene blue. If this were the case, it would be most difficult to explain the relative inertness of malonic or glutaric acids or of substances which are far more prone to oxidation *in vitro* than succinic acid. Succinic acid (or its hydrogen atoms) must be activated prior to reaction, and it is usually assumed that the activation is due to the formation of some unstable complex molecule with the enzyme, this unstable molecule having the effect of rendering the hydrogen atoms of its succinic acid moiety labile. What happens, then, when fumaric acid is activated to become a hydrogen acceptor? (It oxidises leucomethylene blue at p_H 7·4 and 45° in presence of *B. coli* and not in its absence.) If vaguely we comprehend that the lability of hydrogen in succinic acid may be due to combination with an enzyme, it is with much less clearness that we can conceive of a change in fumaric acid, due to combination, which will result in the latter's oxidising power.

Again it is difficult to imagine that there exists a specific enzyme related

to chlorate, especially as this substance, after reduction in presence of *B. coli*, gives rise to chlorite which is extremely toxic to the organism.

Yet if the specific enzyme hypothesis be incorrect or unsatisfactory, what is the explanation of the high reducing power *in vivo* of succinic acid compared with that of glutaric acid, of alanine compared with glycine, of glutaminic acid compared with aspartic acid, of acrylic acid with propionic acid, of lactic acid with glycollic acid and so on?

The site of activations due to bacteria.

The site of reduction of the dyestuff (*e.g.* methylene blue) cannot be outside the organism (*i.e.* in the medium), for if this were so the enzyme responsible for reduction would be extracellular and all observations show that such enzymes are not formed. For instance the filtrates of media on which the organism has been growing, and the solutions obtained after the washing of bacteria with slightly alkaline buffer solution, hypertonic or normal saline solutions, distilled water, etc. give no indication of the presence of a "hydrogen transportase."

If the site of reduction be within the bacterial cell then the cell membrane must be permeable to both the reactants, methylene blue (M.B.) and the hydrogen donator (D.).

Assuming the cell membrane to be composed entirely of lipoid material, then from Loewe's work [1912] we know that M.B. will be adsorbed and not dissolved, *i.e.* that M.B. will not proceed through the membrane. On the other hand if the membrane be not entirely of lipoid nature diffusibility of the M.B. is possible. We will assume that diffusion of the dyestuff through the membrane occurs. Then the rate of reduction of M.B. will depend on the slower of the two processes, (1) the diffusion of M.B. into the cell, (2) the reaction of M.B. with D. inside the cell.

If the rate of diffusion be slower than the chemical reaction, then the temperature coefficient of the reduction will be that of the diffusion process. Experiment shows that the coefficient is too high; it is 2·1 between 30° and 60° for succinic acid and methylene blue in presence of *B. coli* [Quastel and Whetham, 1924]. It follows that the chemical process must be slower than the rate of diffusion of M.B. into the cell.

Again the rate of diffusion of M.B. will be proportional to the difference between the concentrations of M.B. in the cell and of M.B. in solution. Hence if the reduction of M.B. in the cell be so fast that only a minute quantity of M.B. be present in the cell at any moment then the velocity of its reduction will be approximately proportional to its concentration outside the cell. Experiment shows that the rate of reduction is for the most part linear (see Fig. 1), *i.e.* it is independent of the concentration of M.B. Hence the rate of reduction cannot be governed by the rate of diffusion of M.B. into the cell (even at the low concentration of 1 in 100,000) and must therefore be determined by the slower chemical process.

From this it is possible to estimate the lower limit of the rate of diffusion of M.B. into the cell, under the conditions normally employed. We will take the following figures of a single experiment: in presence of 1 cc. *B. coli* suspension containing approximately 5×10^6 cells, 1 cc. of 1/5000 sol. of M.B. was reduced in 10 minutes at p_H 7·4 and 45° the total volume being 6 cc. and 1 cc. $M/20$ formic acid (as neutral salt) being the hydrogen donator.

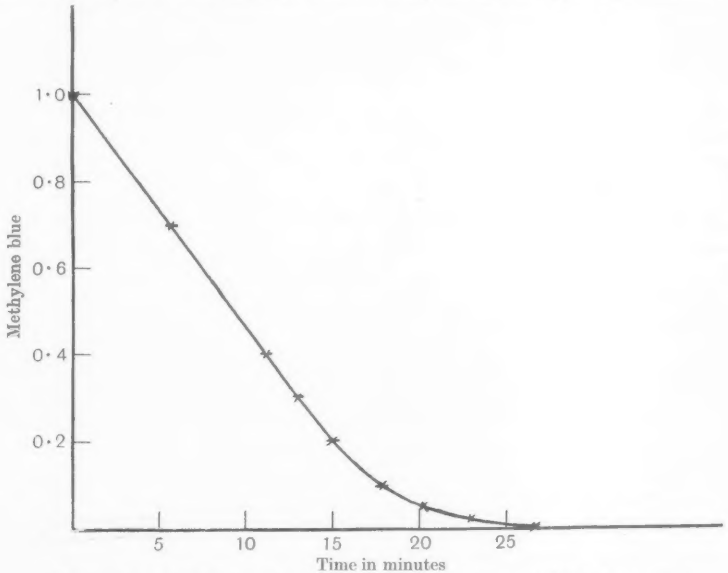


Fig. 1. Rate of reduction of methylene blue by succinic acid $M/140$ in presence of *B. coli*. ($p_H = 7·2$.)

The concentration of M.B. is 1 g. in 30,000 or 1 g. mol. in 9,600,000 cc. (since the molecular weight of methylene blue is approximately 320), *i.e.* $\frac{60·6 \times 10^{22}}{9·6 \times 10^6}$ molecules per cc. ($60·6 \times 10^{22}$ being the Avogadro constant) which is $6·3 \times 10^{16}$. Then the number of M.B. molecules reduced in 1 min. per unit cell is approximately $\frac{6 \times 6·3 \times 10^{16}}{5 \times 10^6 \times 10} = 7·5 \times 10^9$. If the reduction therefore occurs within the cell the minimum rate of diffusion of M.B. into the cell will be $7·5 \times 10^9$ molecules per min.

Taking a cell of *B. coli* as a cylinder 3μ long and diameter $0·5\mu$, its volume is $\frac{\pi 3\mu^3}{16} = 0·6\mu^3 = 0·6 \times 10^{-12}$ cc. The total number of M.B. molecules within the cell when the concentration of dyestuff is equal on both sides of the membrane is $0·6 \times 10^{-12} \times 6·3 \times 10^{16} = 3·8 \times 10^4$.

Hence the maximum time taken for M.B. to diffuse into a *B. coli* cell to reach a concentration within the cell equal to that outside is $\frac{3·8 \times 10^4}{7·5 \times 10^9}$ mins. which is approximately $\frac{3}{10,000}$ of a second.

In calculating this figure no account has been taken of the fact that M.B. molecules can pass out of the cell as well as pass in. Correction for this would make the maximum time of diffusion, *i.e.* $\frac{3}{10{,}000}$ of a second, smaller.

This means, if we accept the view that the whole of the reduction occurs within the cell, that immersion of a suspension of *B. coli* (freshly grown) into a dilute solution of methylene blue (1/30,000) would be followed by an extremely rapid penetration of the dyestuff into the cell. When it is considered that in vital staining dilute solutions of methylene blue are ineffective and strong concentrations must be used [see v. Möllendorf, 1921] and that no penetration of methylene blue from dilute solution into living yeast cells has been observed, although these cells can effect reduction in presence of donators, it appears to be most unlikely that such rapid diffusion of methylene blue into *B. coli* actually takes place.

It is more in harmony with the facts to conclude that the site of reduction of M.B. is not wholly within the cell and is therefore at the outer cell surface.

The question now arises as to the site of activation of the substrate. Assuming for the purposes of argument that the cell is permeable to the substrate it is clear that there exist the following three possibilities: (1) that activation occurs at some structure (or surface) within the cell; (2) that activation is due to combination with some protoplasmic constituent (or enzyme); (3) that activation occurs at the cell surface. Now it is unlikely that the reduction of M.B. occurs within the cell. Hence even if activation of the substrate does occur at some intracellular structure the fact that the molecule will become "normal" out of contact with the structure makes it improbable that such an activation would account for reduction of the dye at the cell surface. If, on the other hand, the activation were due to the formation of an unstable complex molecule (enzyme and substrate) then, since reduction does not occur at the cell's inner surface, the cell membrane must be permeable to the complex molecule and reduction must be effected at the outer surface. But if such permeability were possible we should expect to find the "reducing enzyme" in a broth medium in which bacteria have grown and from which they have been removed. Attempts to find such a soluble reducing enzyme have entirely failed. Again the sterile filtrates of bacteria which have been disintegrated can effect no reduction of methylene blue in presence of such active donators as succinic and formic acids[1].

It is possible to conceive of a reduction occurring through the membrane, the M.B. forming a layer on the outer side of the membrane, the complex molecules (substrate and enzyme) forming a layer on the inner side, and reaction occurring as in the oxidation, through an artificial membrane, of fumaric acid by ferric hydroxide [Girard, 1925]. Now it may be shown (see

[1] I owe much to the kindness of Dr D. Thomson of the Pickett-Thomson Laboratory who has disintegrated suspensions of bacteria for me in his special apparatus. The p_H of *B. coli* during disintegration was not allowed to exceed 8·1.

Table I) that the velocity of reduction of M.B. due to a mixture of two donators (say succinic and lactic acids) is, within the limits of experimental error, the sum of the velocities due to each donator. Thus if we take a concentration of a donator so large that an increase in concentration appears to have little or no effect on the velocity of reduction, the addition of another donator makes

Table I.

Full details of the experimental technique are to be found in previous papers [Quastel and Whetham, 1925, 1, 2]. The following point in technique is of use: when an organism is itself highly reducing, contact for half an hour at room temperature with 2 % saline with subsequent centrifuging and suspension in normal saline is beneficial. The activating powers of the organism are apparently not impaired by this treatment.

Each vacuum tube contained 2 cc. phosphate buffer, 1 cc. 1/5000 M.B., organism and donator, the total volume being made up to 7 cc. with water. Reductions were carried out *in vacuo* at 45°. Calculated times of reduction for mixtures of donators were obtained by considering rate of reduction as linear (Fig. 1); no correction has been made for divergence from linearity towards the end of reduction. *Coli* 1, *coli* 2, etc. are different suspensions and strains of *B. coli*; results with *coli* 1 can be compared strictly quantitatively with one another but only qualitatively with results with *coli* 2, etc. All substances under investigation were brought to p_H 7·2 by sodium hydroxide or hydrochloric acid. Controls with all organisms (absence of donators) did not reduce within two hours. The following are typical results.

Organism	p_H	Donators	Reduction time (observed)	Reduction time (calc.)
0·5 cc. *Coli* 1	7·2	Galactose *M*/280	24′ 30″	
,, ,,	,,	Acrylic acid *M*/70	11 25	
,, ,,	,,	Galactose *M*/280 + acrylic acid *M*/70 ...	8 30	7′ 50″
1 cc. ,,	,,	Mannitol *M*/35	11 50	
,, ,,	,,	,, *M*/140	14 30	
,, ,,	,,	Allyl alcohol *M*/46·6	19 5	
,, ,,	,,	,, *M*/35	19	
,, ,,	,,	Mannitol *M*/140 + allyl alcohol *M*/46·6 ...	8 50	8 15
,, ,,	,,	Succinic acid *M*/47	14 30	
,, ,,	,,	Mannitol *M*/140 + succinic acid *M*/47 ...	7 45	7 15
0·5 cc. *Coli* 2	7·4	Alanine *M*/13	52	
,, ,,	,,	,, *M*/6·5	45 20	
,, ,,	,,	Succinic acid *M*/130	17 35	
,, ,,	,,	Alanine *M*/13 + succinic acid *M*/130 ...	11 20	13 5
0·5 cc. *Coli* 3	7·4	Succinic acid *M*/130	10 10	
,, ,,	,,	Oxalic acid *M*/6·5	No reduction	
,, ,,	,,	Succinic acid *M*/130 + oxalic acid *M*/6·5	11	10 10
0·2 cc. *Coli* 4	7·4	Succinic acid *M*/120	20	
,, ,,	,,	Glycerol *M*/5·5	26 30	
,, ,,	,,	Succinic acid *M*/120 + glycerol *M*/5·5 ...	14	11 30
,, ,,	,,	Allyl alcohol *M*/12	3 30	
,, ,,	,,	Succinic acid *M*/120 + allyl alcohol *M*/12	2 55	3
0·5 cc. *Coli* 5	,,	Succinic acid *M*/140	19	
,, ,,	,,	Lactic acid *M*/140	7 10	
,, ,,	,,	Formamide *M*/1050	4 20	
,, ,,	,,	Succinic acid *M*/140 + lactic acid *M*/140	6 20	5 15
,, ,,	,,	Succinic acid *M*/140 + formamide *M*/1050	3 25	3 30

a definite increase in velocity of reduction. In a word, each donator appears to act as though the other were absent[1]. If then we are to imagine the reducing effect of, say, a high concentration of succinic acid to be due to the formation of a layer of the succinic acid-complex molecules at the inner

[1] Whether this is strictly true for all donators or at all concentrations of the donators cannot, at present, be definitely stated. Clearly it cannot be strictly true in the case of two donators where one is powerfully adsorbed by the cell and the other not.

surface of the membrane it is difficult to see how, say, lactic acid-complex molecules effect their reduction independently of the layer of succinic acid molecules. Again, this view entails so many questionable assumptions (*e.g.* that the cell is permeable to every substance which is activated, that the velocity of diffusion of the substrate into the cell is greater than the chemical process of reduction within the cell, that there exist numerous intracellular specific enzymes) and appears to be so inadequate to explain such well-known facts of biological oxidation as asymmetric oxidation of the double bond, and β-oxidation, that it would be pointless at present to go any further with it.

Summing up, it seems justifiable to assume that not only the site of reduction of M.B. but also the site[1] of activation of the substrate molecule is at the cell surface.

Some considerations of cell surfaces.

In any growing organism we may assume that there exists in each cell a definite and ordered series of reactions which is characteristic of the organism. If this were not the case there could be none of the fixity or specific nature of organisms which is their characteristic feature. The well-known facts with regard to chromosomes, for instance, clearly indicate the ordered series of events within the cell.

The constitution of the cell membrane will be as characteristic a feature of the organism as any structure within the cell [see also Leathes, 1925]. It is sure, too, from the work of Hardy, Langmuir, Harkins and Adam on the orientation of molecules at an interface, that the membranes will have a definite molecular structure, the molecules composing the membrane being orientated with regard to one another in a particular way dependent upon their chemical nature and upon the character of the phases on either side of the membrane. Now since the chemical constitution of the membrane is, probably, very complex and to some extent fluctuating during growth it follows that its molecular structure cannot be expected to evince a homogeneity or symmetry. We should expect, rather, that certain parts of the surface would be occupied by certain molecules or groups of molecules and other parts by other molecules and so on, so that if it were possible to make instantaneous observations at discrete points of the surface it would be found that there existed an irregular distribution of groups, and that the surface, in fact, exhibited a "geography." Doubtless this "geography" or structure will change its form as the cell grows and divides, but so long as the membrane remains intact and suffers no profound chemical change this structure will remain, as a whole, the same and a characteristic feature of the cell.

Associated with certain molecules or groups of molecules orientated in

[1] Or perhaps, better, that *a* site of activation is at the cell surface; for there is no evidence against an activation occurring at an intracellular surface or structure. Such an activation would, however, as pointed out, be ineffective in the case of reduction of M.B. Disintegration of bacteria besides breaking up the cell surface will also destroy intracellular surfaces.

the membrane, there will exist electric fields whose nature will be dependent on the nature of the groups which give rise to them. Some of these fields will be very powerful, due either to certain active radicles or groups or to the juxtaposition or association of certain molecules or groups; others, on the other hand, will, for the same reasons, be very weak. Hence we may imagine scattered over the cell surface many locally intense electric fields, these being a function of the "geography" of this surface. The fields will be modified by any chemical or physical changes which affect the structure of the membrane. The point to which I desire to give most emphasis is the local nature of these electric fields of varying intensity; at certain parts of the surface there may be most powerful fields, although the average field taken over the whole surface may be very weak[1].

Since the membrane structure will be expected to vary somewhat with functional activity we may expect some alteration of the electrical condition of the surface and hence of the *total* surface charge. Hardy and Harvey [1911] from experiments with *Gonium*, *Vorticella* and *Amoeba* incline to the view that surface charge does vary with variations in the state of activity of the living cell. But even if the total surface charge alters, we may still expect the presence of local fields such as have been pictured above.

It is from a consideration of the effects induced in a molecule by the application of an external electric field that I would suggest that an interpretation of the mechanism of activation is forthcoming.

If we are to ascribe the activating powers of an organism to the presence of local intense electric fields, it would follow that, since the surface structure of an organism will fluctuate to some extent during growth, there will exist differences in activating power between one cell and another. Since, however, in experiments with *B. coli* and M.B., some millions of cells are used for each reduction, it is clear that the average activating power of the organism is observed. This average activating power will be the same for any strain of the same organism which has been grown and "handled" in the same way; it will vary to some extent with different strains and it would not be expected that the average activating power of one organism would be identical with that of a totally different organism.

[1] It is important to realise the difference between a surface structure such as has been pictured and, let us say, that of a protein such as caseinogen or albumin. In the former the molecules—or conglomerates of molecules—are in a state of very great complexity due to the juxtaposition of substances which are continually being produced by an actively growing cell. Certain associations of such substances will result in the production of unstable structures—structures of high potential energy—which only exist while the conditions, which make such associations possible, obtain. Such structures as these, I imagine, are the sources of the most powerful electric fields. Molecules such as albumin or caseinogen are relatively stable units compared with these structures, and in a surface composed entirely of albumin or caseinogen a distribution of intensely active centres, which is characteristic of cellular (and intracellular) surfaces, does not exist.

THE MECHANISM OF ACTIVATION.

Let us consider the effect of an external electric field upon the unsaturated linkage. In Sir J. J. Thomson's words [1923, 1] we will "suppose that there is a double bond between two carbon atoms $C_2{=}C_1$, the octets of electrons round C_1 and C_2 having four electrons in common situated between C_1 and C_2. If an external electric field acts on the molecule, tending to make electrons move from left to right, some of the electrons held in common may be so far displaced from C_2 and towards C_1 that they can no longer be regarded as shared with C_2. If two of these are displaced far enough for this to happen, the octet round C_1 will be intact and C_1 will be saturated, whilst the octet round C_2 will be reduced to a sextet, so that C_2 will be unsaturated and chemically active: there are still two electrons left between C_1 and C_2 to form a single bond binding C_1 and C_2 together." The effect of the field, then, is to produce a shift of the electrons from their normal paths to new orbits, and of course, when out of contact with the field, the orbits will revert to normal. The same force, however, which produces a shift in the electrons will also alter the relative positions of the protons (hydrogen ions). Hence, interpreting in chemical terms the effect of an external or polarising field upon the double bond $—C_1H{=}C_2H—$, there will be a shift of a proton from C_1 towards C_2 or *vice versa*, the extent of the shift depending upon the nature and strength of the external field. The greater the shift the more unsaturated and chemically active will one end of the bond become. We shall identify a molecule in such a polarised condition as one in an activated state, the polarising field being the activating source.

The following equations represent the change after maximum activation:

$$\underset{\text{normal}}{—CH{=}CH—} \rightarrow \underset{\text{activated}}{—\overset{\vee}{C}—CH_2—}$$

$$\underset{\text{normal}}{—CH{=}O} \rightarrow \underset{\text{activated}}{—\overset{\vee}{C}—OH}$$

$$\underset{\text{normal}}{—CH{=}N—} \rightarrow \underset{\text{activated}}{—\overset{\vee}{C}—NH—}$$

Now the actual shift of the proton will be determined, not only by the external electric field but by the electrical condition of the molecule itself, *i.e.* by the nature of the groups composing the molecule. Thomson [1923, 2] has shown that the "electrical effect of replacing an atom of hydrogen by another atom or radicle may be represented by the introduction of an electric doublet at the hydrogen atom." If the radicle forms a system requiring an electron to complete its octet the positive end of the doublet will be towards the molecule with which the radicle is combined. Thus if such a radicle replaces a hydrogen combined to a carbon atom the positive end of the doublet will be towards the carbon and the electrical effect at the carbon atom will be to drive away positive electricity and attract negative. The reverse effect holds with a radicle in which there is one electron over after providing for

complete octets. Table II summarises the electrical effects of various radicles at the C atom situated in the α-position to the radicle.

Table II.

Radicle	No. of electrons	Electrons necessary to complete octet	Effect at the α-C atom	Polarity
$-CH_3$	7	+1	Positive electricity repelled	Non-polar
$-OH$	7	+1	,,	Polar
$-NH_2$	7	+1	,,	,,
$-CH_2COOH$	23	+1	,,	,,
$-COOH$	17	−1	Positive electricity attracted	,,
$-CONH_2$	17	−1	,,	,,
$-COCH_3$	17	−1	,,	

The aldehyde group [Thomson, 1923, 2] and the group —CO.COOH act in the same sense as the carboxyl.

Let us now consider the effect of activating the structure A.CH=CH.X where A is assumed to have no directive influence on the movement of the proton. Assuming maximum activation to have occurred, three typical results are shown in Table III.

Table III.

X	Normal form of molecule	Activated form of molecule
—COOH	A—CH=CH—COOH	A—$\overset{\vee}{C}$—CH_2.COOH
$-CH_3$	A—CH=CH—CH_3	A—CH_2—$\overset{\vee}{C}$—CH_3
$-CH_2COOH$	A—CH=CH—CH_2COOH	A—CH_2—$\overset{\vee}{C}$—CH_2COOH

The effect of a carboxyl group at X is to produce an electric field at the α-carbon atom which attracts positive electricity. The same effect will to some extent affect the β-carbon atom also and possibly render this more liable to attract positive electricity than it was before the introduction of the carboxyl group. The field, however, is far stronger at the α-C atom and hence the shift of the proton will be from the β-C to the α. This shift will render the β-C chemically unsaturated, whilst the α-C becomes relatively saturated. Hence when oxidation occurs *after* activation, a symmetrical oxidation (or addition of negative electricity at the α- and β-C atoms) will not take place; only a β-oxidation is possible. On the other hand if a $-CH_3$ group is present at X the movement of the proton ($H^{\bullet}$) after activation will be from the C in the α-position to that in the β, and the α-C will now attract negative electricity. (The repelling effect of the CH_3 group on hydrogen at the adjacent C is seen in acetoacetic ester, which exists normally to the extent of 98 % in the keto form [Knorr, 1911].)

Consider, now, the cases of fumaric acid and crotonic acid. The normal acids COOH.CH=CH.COOH and CH_3.CH=CH.COOH will, after activation, become COOH.$\overset{\vee}{C}$.CH_2.COOH and CH_3.$\overset{\vee}{C}$.CH_2.COOH respectively. In the case of activated fumaric acid there is a field acting at the unsaturated

carbon which attracts positive electricity (H') and is due to the adjoining carboxyl group. Hence activated fumaric acid will tend to attract hydrogen. In the case of crotonic acid the field acting at the unsaturated C due to $—CH_3$ will result in a strong attractive effect for negative electricity. Hence we should not expect either of the crotonic acids to be such powerful hydrogen acceptors as fumaric acid, and they should be easily oxidised, after activation, to acetoacetic acid. Experiment shows this to be true (see Table IV), *iso*crotonic acid acting as a fair hydrogen donator in contrast to fumaric acid. The ease with which the crotonic acids are oxidised to acetoacetic acid is shown by the following experiment. In tubes are placed 2 cc. phosphate buffer p_H 7·4, 2 cc. $M/2$ crotonic or *iso*crotonic acid (as the neutral sodium salt) and 3 cc. of a fairly thick suspension of *B. coli*. The tubes are allowed to stand 24 hours at 37° after which they are tested for the presence of acetoacetic acid by the usual nitroprusside reaction. A very good positive test results in the cases of both acids. Better yields of acetoacetic acid are obtained by adding to the mixture of buffer, crotonic acid and *B. coli*, 2 cc. $M/20$ sodium nitrate solution. In the presence of nitrate, an intense acetoacetic acid test may be obtained, even in the absence of oxygen.

Methylene blue as a hydrogen acceptor.

The value of methylene blue as a hydrogen acceptor lies not so much in the fact that it is "easily" reduced or that it gives a colourless leuco-compound, but that it does not require activation by an enzyme to exert its oxidising power. It is typical of the substances which give oxidation-reduction potentials and may be regarded as "active" *in vitro*. Thus we can use methylene blue as a standard for comparing the activities of various molecules in presence of an activating source (*e.g. B. coli*). But if a substance in presence of *B. coli* does not perceptibly reduce methylene blue or oxidise its leuco-compound, it does not necessarily follow that the substance has not been activated. We may say that it is inert compared with another substance but not necessarily as inert as it is in absence of the activating source. It is, moreover, clear that a substance apparently inert towards methylene blue may reduce some other hydrogen acceptor. It should be possible, therefore, with a series of substances of known oxidation-reduction potentials to estimate the activation of a substance by an organism by determining at what potential hydrogen donation is just observable. A reliable potentiometric method would, of course, accomplish the same end.

In the activated structure $—\overset{\vee}{C}—CH_2—COOH$, it is necessary to compare the relative affinities of this structure and of, say, methylene blue for hydrogen. The latter will go to the molecule in which the affinity is stronger and if this be methylene blue, reduction of the dyestuff and oxidation of the structure will occur. Thus

$$—\overset{\vee}{C}—CH_2—COOH + 2OH.H + MB \rightarrow —CO.CH_2—COOH + MBH_2 + H_2O.$$

If the affinity be stronger with the activated structure, this will act as an oxidising agent towards leuco-methylene blue. Thus

$$—\overset{\vee}{C}—CH_2—COOH + MBH_2 \rightarrow —CH_2—CH_2—COOH + MB.$$

With "active" oxygen as an acceptor, it is possible that the affinity of this for hydrogen is greater than that of the activated structure; so that finally we may have a structure which is oxidised by "active" oxygen and yet itself acts as an oxidising agent towards leuco-methylene blue. Hence the dual nature of the activated double bond. Fumaric acid is a good instance [cf. Quastel and Whetham, 1924; Quastel, Stephenson and Whetham, 1925].

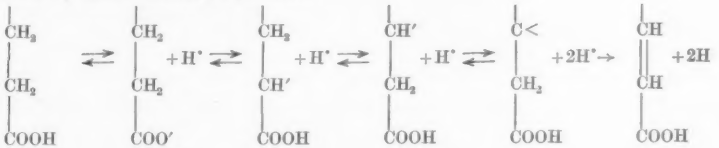
The activation of the saturated bond.

Consider the structure $—CH_2—CH_2—COOH$. It is clear that in this case somewhat different considerations must be taken into account from those in the case of the corresponding unsaturated structure. It is necessary, with both structures, to consider in more detail the condition of the carboxyl group. We may assume that we have to deal normally with the ionised carboxyl (in feeding experiments or experiments with bacteria an acid is given as the neutral salt). The charged carboxyl group has an effect on the hydrogen attached to the α-C and it is reasonable to suppose that such a hydrogen will be rendered mobile and will oscillate between the α-C and one of the oxygen atoms in the carboxyl group. Thus the normal forms of the structure $—CH_2.COOH$ will be represented by the equilibria:

$$\underset{\text{I}}{—CH_2—COOH} \rightleftarrows \underset{\text{II}}{—CH_2—COO'} \overset{+H^{\cdot}}{\rightleftarrows} \underset{\text{III}}{—CH'—COOH} + H^{\cdot}$$

Now the movement of the α-H (proton) is much more likely to occur in a saturated structure (I) than in the corresponding unsaturated structure $=CH—COOH$, for in the latter the attractive effect of the α-C for positive electricity will oppose the attractive effect of the oxygen in the carboxyl. If, however, there be attached to the α-C atom a grouping which has the effect of repelling positive electricity at this atom, a shift or movement of the hydrogen will take place. In a saturated structure (I) movement of an α-H occurs since the demand of the α-C for positive electricity is, at any rate partially, satisfied by the presence of the second hydrogen, which remains more or less fixed.

Now the complete picture of activation of $—CH_2—CH_2COOH$ is represented by the following equilibria:



On activating III a proton will move in the direction of the atom where the attraction for positive electricity is strongest, *i.e.* from the β- to the α-C; IV will undergo the same process as I (*i.e.* I $\rightleftarrows$ II $\rightleftarrows$ III) and the final activated form will be V. But V, as we have already seen, is the activated form of VI, so that if there be present in the original system an activating source, the structure I and a hydrogen acceptor whose affinity for hydrogen is greater than that of V, reaction will occur to produce VI and the reduced form of the hydrogen acceptor. This scheme I $\rightarrow$ VI + 2H represents, in the clearest way, Wieland's theory of activation of hydrogen.

Further considerations on the influence of radicles.

It is clear that not only the electrical effects due to radicles, but the effects of the polarities of radicles must be taken into account—polarity being used, here, in the sense employed by Hardy and Langmuir. Examples of polar groups, *i.e.* groups polar to or "dissolved" by water are the carboxyl, hydroxyl and amino groups. Examples of non-polar groups, *i.e.* groups directed to non-aqueous solvents, are methyl, or ethyl groups, or fatty chains generally.

If the cell membrane be considered as a non-aqueous phase it follows that the presence of non-polar groups in a substrate molecule will tend to produce an adsorption of the molecule at the cell interface. A blocking—or partial blocking—of the membrane will ensue, the extent of which will depend upon the nature of the non-polar and polar moieties of the molecule. This will result in retardation of the velocity of a process which depends upon the activation, by the cell, of one or both of the reacting molecules. Thus methyl, ethyl and propyl alcohols retard and inhibit reductions dependent on the presence of *B. coli*, the inhibition being greatest, for equivalent concentrations, with propyl alcohol and least with methyl alcohol, and total inhibition of the reducing effect of different donators by a given alcohol requiring approximately, as we should expect, the same concentration of the alcohol [Quastel and Whetham, 1925, 1][1].

We should expect a hydrogen donator possessing non-polar groups to inhibit its own reductions—the effect being observed as the concentration of the donator is changed. This is true in the cases of acetaldehyde (Table IV), and of ethyl and propyl alcohols and of *iso*butyric, *iso*valeric and caproic acids [Quastel and Whetham, 1925, 1].

The effect, then, of a non-polar group, whatever its electrical effect, is to produce an apparent diminution in the activity of the molecule of which it is a constituent.

The double bond is interesting in this connection. Langmuir [1917] has shown that it is polar in character. With this is consistent the fact that allyl

[1] An error was made in the curve Fig. 6 [Quastel and Whetham, 1925, 1]. The reduction times for 0·25 *Coli* should read 8′, for 0·5 *Coli* 4′ 15″ and 1·0 *Coli* 2′ 15″, instead of 8′ for the three amounts of organism.

alcohol, which is a good donator, does not apparently retard either its own reduction or those of other donators (Table IV).

Again phenol, with three double bonds, has but a slight inhibitory effect on reductions due to succinate, whereas the corresponding saturated compound, cyclohexanol, has a most powerful inhibitory effect. Toluene, in which the polar OH of phenol has been replaced by the non-polar CH_3, inhibits more vigorously than phenol [Quastel and Whetham, 1925, 1]. Generally speaking, were it desired to find a vigorous inhibitor of reactions dependent on the activity of, or taking place at the surface of, a cell, I should seek a molecule possessing powerful non-polar groups, and yet sufficient polarity to make it possible for it to go into aqueous solution. Such an inhibitor would, also, act as an antiseptic.

The polar effects of various radicles are summed up in Table II (p. 175). It is necessary also to bear in mind (1) the basic nature of the amino-group and the acidic nature of the carboxyl; (2) the ability of such radicles as CH_3, OH, NH_2, to part with hydrogen and hence to change their polar and electrical characters.

Let us, now, apply these considerations to substances for which experimental details have been obtained.

Table IV.

Conditions as in Table I.

The following are typical of a large number of results:

Organism	p_H	Donator	Reduction time
5 cc. *coli* 6	7·4	Succinic acid *M*/140	27′
,, ,,	,,	Thiodiglycollic acid *M*/52·6	Not reduced in 2 hrs.
,, ,,	,,	,, *M*/26·3	,,
1 cc. *coli* 7	7·2	Acrylic acid *M*/140	,,
,, ,,	,,	,, *M*/80	8′
,, ,,	,,	,, *M*/70	5
,, ,,	,,	,, *M*/35	4′ 20″
1 cc. *coli* 8	7·2	Allyl alcohol *M*/140	37 45
,, ,,	,,	,, *M*/70	26 30
,, ,,	,,	,, *M*/35	19
1 cc. *coli* 9	7·2	β-Hydroxybutyric acid *M*/140	Not fully reduced in 2 hrs., appears to reach an equilibrium after 3′ which remains stationary for 1 hr.
,, ,,	,,	,, *M*/112	Indication of slight reversal after 4′, 75 % of the methylene blue having been reduced. Complete reduction in 87′.
,, ,,	,,	,, *M*/93	Complete reduction in 5′, followed by slight reversal after 21′.
1 cc. *coli* 10	7·4	Succinic acid *M*/140	11′ 30″
,, ,,	,,	Pyrotartaric acid *M*/70	77
,, ,,	,,	,, *M*/14	71
,, ,,	,,	Tartaric acid *M*/14	65
,, ,,	,,	,, *M*/70	Not reduced in 2 hrs.
,, ,,	,,	Tricarballylic acid *M*/105	65′
,, ,,	,,	,, *M*/42	64
,, ,,	,,	,, *M*/21	62
,, ,,	,,	Pyromucic acid *M*/70	23
,, ,,	,,	,, *M*/28	21′ 30″
,, ,,	,,	,, *M*/14	18 30
,, ,,	,,	Oxalic acid *M*/14	Not reduced in 2 hrs.
1 cc. *coli* 11	7·4	Succinic acid *M*/140	7′

Table IV (*continued*).

Organism	p_H	Donator	Reduction time
1 cc. *coli* 11	7·4	*n*-Crotonic acid *M*/14	Not reduced in 2 hrs.
,, ,,	,,	,, *M*/7	,,
,, ,,	,,	*iso*Crotonic acid *M*/28	,,
,, ,,	,,	,, *M*/14	22′
,, ,,	,,	,, *M*/7	14
,, ,,	,,	Succinic acid *M*/140 + *n*-Crotonic acid *M*/14	7′ 30″
,, ,,	,,	Allylamine *M*/14	Partial reduction only in 2 hrs.
,, ,,	,,	,, *M*/7	,,
1 cc. *coli* 12	7·4	Formamide *M*/5650	58′ 10″
,, ,,	,,	,, *M*/2825	56 15
,, ,,	,,	,, *M*/1883	33
,, ,,	,,	,, *M*/1412	11 30
,, ,,	,,	,, *M*/1130	5 10
,, ,,	,,	,, *M*/565	4 10
,, ,,	,,	Laevulinic acid *M*/35	34
,, ,,	,,	,, *M*/70	36
,, ,,	,,	Succinic acid *M*/140	12 30
,, ,,	,,	Acetic acid *M*/14	25
,, ,,	,,	Acetamide *M*/10·5	No reduction in 2 hrs.
,, ,,	,,	Succinimide *M*/140	90′
,, ,,	,,	,, *M*/35	60
,, ,,	,,	Methylamine *M*/22	Not reduced in 2 hrs.
,, ,,	,,	Ethylamine *M*/28	,,
,, ,,	,,	Acetaldehyde *M*/105	48′ 30″
,, ,,	,,	,, *M*/42	60 20
,, ,,	,,	,, *M*/21	72 20
,, ,,	,,	,, *M*/10·5	Not reduced in 2 hrs.
2 cc. *coli* 13	7·4	None (control)	22′ (a highly reducing organism was chosen)
,, ,,	,,	Glutaconic acid *M*/70	17′
,, ,,	,,	,, *M*/14	15
,, ,,	,,	Mesaconic acid *M*/21	17′ 30″
,, ,,	,,	Citraconic acid *M*/14	18
,, ,,	,,	*iso*Crotonic acid *M*/14	8
,, ,,	,,	Fumaric acid *M*/70	Not completely reduced in 2 hrs.
1 cc. *coli* 14	7·4	0·1 cc. acetonyl acetone	,,
,, ,,	,,	0·5 cc. ,,	,,
,, ,,	,,	1·0 cc. ,,	,,
,, ,,	,,	Succinic acid *M*/140	25′
,, ,,	,,	Glutaconic acid *M*/28	Partial reduction in 2 hrs.
,, ,,	,,	Mesaconic acid *M*/28	Not reduced in 2 hrs.
,, ,,	,,	Citraconic acid *M*/28	,,
0·5 cc. *coli* 15	7·4	Laevulinic acid *M*/14	45′
,, ,,	,,	Acetonyl acetone *M*/4	Partial reduction in 2 hrs.
,, ,,	,,	Sodium chloride *M*/14 (salt control)	No reduction in 2 hrs.

The activation of unsaturated acids.

Acrylic acid[1] is a good hydrogen donator in presence of *B. coli* (Table IV). Both *n*-crotonic (M.P. 72°) and *iso*crotonic acids are activated by the organism, the latter acting as a fair hydrogen donator (Table IV).

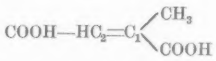
The question of the effect of *cis-trans*-isomerism now arises. It must be immediately clear that the electrical conditions within a *cis*-molecule cannot be identical with those of the *trans*-isomer. Hence the effects of the same activating source on the two molecules will differ; the difference in activation depending upon the extent to which the various groups in the molecules

[1] The reduction experiments with allyl alcohol, acrylic acid, and β-hydroxybutyric acid, mentioned in this paper, were performed in collaboration with Miss M. D. Whetham.

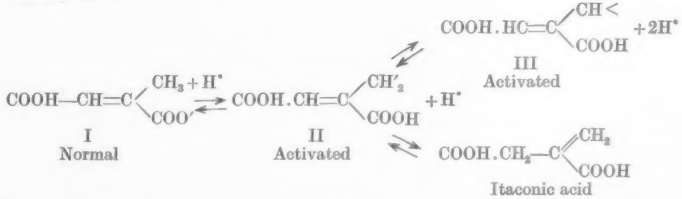
enhance or diminish each other's electrical effects according to their relative positions. Thus the stereo-structure of a molecule plays a most important part in determining its activity in presence of an activating source.

n-Crotonic and *iso*crotonic acids show, very clearly, a difference in hydrogen donating powers. Fumaric and maleic acids exhibit a similar and striking difference (Table IV) [see also Quastel and Whetham, 1924].

In mesaconic and citraconic acids we have our first examples of the antagonistic action of the methyl upon the carboxyl group. Consider the molecule



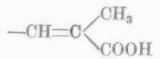
The effect at C_1 of the adjoining carboxyl group is to attract, and of the methyl group to repel positive electricity. Hence, on activation, it is unlikely that a proton at C_2 will move to C_1. In fumaric acid, where there is symmetry of the molecule and no CH_3 effect, the proton can move from C_1 to C_2 or *vice versa*, the positions being equivalent. With mesaconic or citraconic acid there is no proton at C_1 to move towards C_2, and hence, although activation will have made a shift in the electrons of the double bond, we cannot expect these acids, in presence of the same activating source, to exhibit the same reactivity as fumaric acid. At C_1, however, the methyl group is affected by the carboxyl, and the demand of C_1 for positive electricity, which cannot be satisfied by a proton from C_2 owing to the repellent effect of CH_3, will occasion the shift of a proton, after powerful activation, from this group. The following scheme represents the mode of activation of mesaconic acid.



III represents the final activated form following on ionisation of the carboxyl and movement of the β-H in accordance with the representations given earlier. Itaconic acid represents a possible stage after II, in the absence of a hydrogen acceptor. III will become oxidised, in presence of a suitable acceptor, say active oxygen, to the aldehyde, suffer decarboxylation and subsequent oxidation and then proceed through fumaric acid to pyruvic and acetic acids. Thus mesaconic and citraconic acids are liable to attack, the reactive point being the methyl group. Clearly, however, the activation necessary to produce III from I will be much greater than that necessary to produce the active from the normal form of fumaric acid, and only a powerful hydrogen acceptor such as oxygen would be expected to exhibit reactivity with mesaconic acid.

Experiment shows mesaconic and citraconic acids to be relatively inert towards methylene blue in presence of *B. coli* (Table IV). The difference between the two acids is demonstrated by the fact that bacterial growth occurs abundantly on the *trans* and not so well on the *cis* form [Liot, 1923] just as in the case of fumaric and maleic acids[1].

It would be expected from what has been said above that all substances of the type



would, on activation, exhibit a reactivity, at any rate with oxygen, the point of attack being the methyl group. After oxidation, decarboxylation, etc., the final form would be —CH=CH.COOH which would then, on activation, behave in the same way as crotonic or fumaric acid. This is in agreement with general experience on the oxidation *in vivo* of acids with branched chains [Raper, 1914].

We turn, now, to the homologue of fumaric acid—glutaconic acid, I. The activated form is II.

$$\underset{\text{I}}{\text{COOH.CH=CH—CH}_2\text{.COOH}} \rightleftarrows \underset{\text{II}}{\text{COOH.CH}_2\text{—}\overset{\vee}{\text{C}}\text{.CH}_2\text{.COOH}}$$

Since the effect of the $—CH_2.COOH$ moiety is to produce a field at the β-C to repel positive electricity, it would not be expected that II would be a good H acceptor. Experiment (Table IV) demonstrates this. But in considering the production of II another fact must be taken into account. It has been shown by Thorpe and his co-workers that in the three carbon system there is a tendency for a mobile H atom to take up a position on the central C atom of the system, and that the system so produced is relatively stable. Glutaconic acid is, therefore, represented as $COOH.C'H.CH_2.C'H.COOH$ (III), a "semi-aromatic" structure which possesses a considerable measure of stability. It cannot be assumed, therefore, that glutaconic acid will react towards an activating source as readily as, say, fumaric acid. Since III, however, will give rise to a certain amount of I, a slight activation (production of II) will be expected. Dakin [1922], in fact, has found that glutaconic acid gives rise, in presence of muscle, to a small quantity of β-hydroxyglutaric acid under conditions in which fumaric acid is transformed into malic acid to the extent of 70 %. In presence of *B. coli* glutaconic acid appears to be relatively inert (Table IV), a slight hydrogen donating power being observable at high concentration.

The fact that a double bond in a ring can be activated is demonstrated by the activity of pyromucic acid in presence of *B. coli* (Table IV). In a ring, however, which is rendered comparatively stable by tautomerism of hydrogen only a most powerful activation will produce a reactive structure. This fact,

[1] I have not been successful in obtaining any growth of *B. pyocyaneus* on citraconic or maleic acid, although good growth occurs on mesaconic or fumaric acid.

it seems to me, accounts for the relative stability of the benzene ring in the body. Clearly in a compound containing both the benzene ring and a fatty acid chain, the latter will be the susceptible structure.

Activation of the saturated dibasic acids.

The following scheme represents the activation of succinic acid:

$$\underset{\text{I Normal}}{COOH{-}CH_2{-}CH_2{-}COOH} \rightleftarrows H^{\cdot} + \underset{\text{II Normal}}{COOH{-}CH_2{-}CH'{-}COOH}$$

$$2H^{\cdot} + \underset{\text{III Activated}}{COOH.CH_2{-}\overset{\times}{C}{-}COOH} \rightarrow 2H + \underset{\text{Normal}}{COOH.CH{=}CH.COOH}$$

The repelling effect of $COOH.CH_2$— on positive electricity will, after activation of II, result in the production of III, the latter being the activated form of fumaric acid. Now since in succinic acid (1) each of the α-C atoms is equally susceptible to the influence of the neighbouring —COOH and $-CH_2.COOH$ groups, (2) the activated form III readily falls into normal fumaric acid, (3) there are no non-polar groups, we expect the molecule to be a vigorous hydrogen donator—as is the case.

Turning to glutaric acid we expect the activation to proceed on the following lines:

$$\underset{\text{Normal I}}{COOH.CH_2.CH_2.CH_2.COOH} \rightleftarrows H^{\cdot} + \underset{\text{Normal II}}{COOH.CH_2.CH_2.CH'.COOH}$$

$$H^{\cdot} + \underset{\text{Activated III}}{COOH.CH_2.CH'.CH_2.COOH}$$

$$2H^{\cdot} + \underset{\text{Activated IV}}{COOH.CH_2.\overset{\times}{C}.CH_2.COOH}$$

$$2H + \underset{\text{V}}{COOH.CH_2.CH{=}CH.COOH}$$

In II the repelling influence due to $COOH.CH_2$— is at the β-C atom; hence the single H at the α-C will not be made mobile, as in succinic acid, and on activation a proton will move from the β-C to the α-C which is attracting positive electricity. The final activated form is IV, which is the activated form of glutaconic acid. The difference between glutaric and succinic acids is in this way made clear; in the latter either of the α-C atoms becomes unsaturated and reactive, in the former only the β-C. As already pointed out, however, in a three carbon system, the tendency is for mobile hydrogen to take up a position at the central C. Hence it is unlikely that IV will be produced from III except by most powerful activation. We should anticipate, therefore, that glutaric acid would not be a good donator of hydrogen to methylene blue. Experiment shows this to be the case [Thunberg, 1920; Quastel and Whetham, 1925, 1].

We should also expect that oxalic acid and thiodiglycollic acid would be inert as H donators in presence of *B. coli*. This is the case (Table IV) [Quastel and Whetham, 1925, 1].

THE EFFECTS OF SUBSTITUENTS IN SUCCINIC AND GLUTARIC ACIDS.

Consider the structure $COOH.\underset{2}{CH_2}—\underset{1}{CHX}—COOH$ where X is a radicle which produces at C_1 a field repelling positive electricity. After ionisation the structure becomes either

(A) $COOH.\underset{2}{CH_2}—\underset{1}{C'X}—COOH$,

or (B) $COOH.\underset{2}{C'H}—\underset{1}{CHX}—COOH$.

In (A) the attractive field for H at C_1 due to the carboxyl is neutralised (or partially so) by the repelling field due to X. Hence after activation, it is unlikely that a proton will move from C_2 to C_1. In (B) after activation, a proton should move from C_1 towards C_2, the activated structure being $COOH.C'H—C'X.COOH$ (I) in equilibrium with $2H^{\cdot}$.

Now I is the activated form of $COOH.CH{=}CX.COOH$ (II) and if the affinity of I for hydrogen is less than that of methylene blue, II will be formed and reduction of methylene blue will occur. The formation of II would be facilitated if this were rendered comparatively stable by the presence of a tautomeric hydrogen atom, as when X is —OH (and probably $—NH_2$). When X is $—CH_3$ mesaconic or citraconic acid is formed; these acids have already been discussed. It is important to note, however, the difference between —OH (or $—NH_2$) and $—CH_3$. In the case of the former the production of a tautomeric keto-enol body stabilises the molecule II; in the case of the latter, oxidation of $—CH_3$ to —CHO (as with mesaconic acid) occurs with subsequent oxidation and decarboxylation and the final substitution of a H for the $—CH_3$. We should expect, then, the structure

$$COOH.CH_2.CHX.COOH$$

to be a hydrogen donator, but not so powerful as succinic acid because the activity depends mainly upon a shift of a proton from C_1 to C_2, the reverse shift from C_2 to C_1 not occurring.

Experiment shows that where X is $—CH_3$, —OH, $—NH_2$ and $—CH_2COOH$ hydrogen donation occurs generally at fairly high concentration. (For pyrotartaric and tricarballylic acids see Table IV; for details on malic and aspartic acids see Quastel and Wooldridge [1925], Thunberg [1920], Fleisch [1924].)

Malic and aspartic acids need special consideration. Both substances act as weak hydrogen acceptors in presence of *B. coli* [Quastel and Whetham, 1924; 1925, 2]. In the activated structure $COOH.\breve{C}.CH_2.COOH$, two fields act at the unsaturated C, one to attract positive electricity, one to attract

negative. Thus the addition of the elements of water is probable, so that at the activating surface the following equilibrium will ensue:

$$COOH.\breve{C}.CH_2.COOH + H.OH \rightleftarrows COOH.CHOH.CH_2.COOH.$$

The effect of the activating field on malic acid, therefore, is to produce a certain amount of activated fumaric acid which acts as a hydrogen acceptor. Hence we have malic acid, after activation, acting both as a hydrogen acceptor and donator. (Probably the donating properties of the acid are obscured in the experiments with *B. coli* by the accepting powers.) The same effect may hold with aspartic acid and the possibility of an equilibrium existing in presence of bacteria between fumaric acid, ammonia and aspartic acid is now under investigation[1].

It is useful to consider the various reactions to which the activated structure $—\breve{C}—CH_2—$ can give rise. These are:

(1) $—\breve{C}—CH_2— \rightleftarrows —CH=CH—$

(2) $—\breve{C}—CH_2— + 2H \rightarrow —CH_2—CH_2—$

or (3) $—\breve{C}—CH_2— + 2(OH) \rightarrow —C(OH)_2—CH_2 \rightarrow —CO—CH_2—$

and (4) $—\breve{C}—CH_2— + H.OH \rightleftarrows —CHOH—CH_2—$

All these reactions[2], once activation has occurred, may proceed at the same time, the rates of the reactions being dependent upon (1) the extent of activation, (2) the physical and chemical conditions of the environment.

In viewing, then, the oxidation of the structure $—CH_2—CH_2—COOH$, it is only necessary to picture the activated form $—\breve{C}—CH_2—COOH$ as the first step prior to reaction and not necessarily to picture either the β-keto-acid, the β-hydroxy-acid or unsaturated acid as the *first* product of oxidation. These substances may all be regarded, in a sense, as the first products of oxidation.

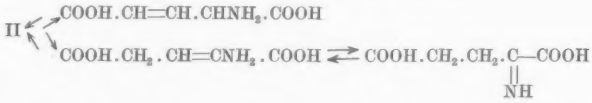
The difference between aspartic and glutaminic acids, *in vivo*, is also made clear. The latter cannot act as hydrogen acceptor since the corresponding unsaturated acid, glutaconic acid—even assuming that an equilibrium does exist between these two acids—is relatively inert as a hydrogen acceptor. Thus, whereas aspartic acid will enable *B. coli* to grow anaerobically upon glycerol, glutaminic acid has no such effect [Quastel and Stephenson, 1925]. Glutaminic acid, on the other hand, should be a fair hydrogen donator, the exceptional condition obtaining in glutaric acid not holding in this case. The amino-acid should react thus:

$$\underset{\text{I Normal}}{COOH.CH_2.CH_2.CHNH_2.COOH} \rightleftarrows \underset{\text{II Final activated form}}{COOH.CH_2.\breve{C}.CHNH_2.COOH} + 2H^{\cdot}$$

[1] The work on this problem, which is being pursued by Mr B. Woolf and the writer, shows, so far, that production of aspartic acid does occur from fumaric acid and ammonia in presence of resting *B. coli*. It is hoped to publish details soon.

[2] Reactions (2) and (3) may proceed simultaneously to produce a Cannizzaro reaction.

II may react subsequently in the following ways:



Experiment shows glutaminic acid to be a good donator [Thunberg, 1920; Quastel and Whetham, 1925, 2]. For similar reasons α-hydroxyglutaric acid should also be a good donator. Thunberg has shown this to be true in the presence of muscle.

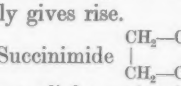
The compound COOH.CHX.CHX.COOH where X is of the type CH_3, OH, NH_2 should be relatively feeble or inert as a H donator[1]. Tartaric acid has been investigated and this substance is only active at high concentrations [Quastel and Whetham, 1925, 1].

The effect of modifying the carboxyl group.

Any modification of the carboxyl group which prevents ionisation of the latter should diminish or entirely remove hydrogen donating power.

Laevulinic acid $CH_3.CO.CH_2.CH_2.COOH$ should be a fair donator of hydrogen for similar reasons to those put forward for succinic acid ($CH_3.CO.CH_2$— acts in the same sense as $OH.CO.CH_2$—but should not be as powerful as succinic acid owing to (1) the asymmetry of the molecule, (2) greater non-polarity. This is the case (Table IV).

Acetonylacetone $CH_3.CO.CH_2.CH_2.CO.CH_3$ in which all ionisation of the carboxyl has been eliminated should be relatively inert. This is found to be true (Table IV), the feeble reductions which are observed with this diketone probably being due to the unsaturated structures to which acetonylacetone easily gives rise.

Succinimide  should also be inert. Experiment shows that only a slight activation occurs at high concentrations; this activity may, indeed, be due to traces of succinic acid produced by hydrolysis (Table IV).

Whilst acetic acid is a fairly good donator, acetamide is inert (Table IV).

α-Ketoglutaric acid should be a good H donator (since COOH.CO— acts in the same sense as COOH—) and that this is so has been shown by Thunberg [1921].

The esterification of the carboxyl group and hence the loss of the power of ionisation of the latter will also "protect" the molecule from oxidation. The stability of glutathione in presence of muscle [Hopkins and Dixon, 1922] is probably to be attributed to this effect: it will be noted that in glutathione the "effective" carboxyl of glutaminic acid is linked with cystine.

[1] Generally speaking when X is OH we should expect the enfeebling effect to be less owing to the relative ease with which H is dissociated from O.

THE ACTIVATION OF BUTYRIC ACID AND THE EFFECTS OF SUBSTITUENTS.

The activation of butyric acid will be represented by the following scheme:

$$\underset{\text{I Normal}}{CH_3—CH_2—CH_2—COOH} \rightleftarrows \underset{\text{II Normal}}{CH_3.CH_2.CH'.COOH} + H^{\cdot} \rightleftarrows$$

$$\underset{\text{III Activated}}{CH_3.CH'.CH_2COOH} + H^{\cdot} \rightleftarrows \underset{\text{IV Final activated}}{CH_3.\check{C}.CH_2.COOH} + 2H^{\cdot} \rightarrow CH_3.CH{=}CH.COOH + 2H$$

Butyric acid should be a good H donator, but owing to the non-polar nature of the propyl group the activation is, apparently, diminished considerably. Experiment shows it to be a donator at high concentration in presence of *B. coli* [Quastel and Whetham, 1925, 1]. If, now, we substitute the hydroxyl (polar) group in butyric acid, a considerable increase in activity should result.

On substituting OH in the β-position the effects may be summarised:

(1) removal of the non-polar influence of the propyl group;

(2) enhanced repulsion of a proton, on activation, from the β- to the α-C, since both CH_3 and OH act in the same sense. Hence we expect β-hydroxybutyric acid to be a good H donator, and to be much more powerful than butyric acid. Experiment shows this to be the case[1] [Thunberg, 1920]; see also Table IV. The arguments which apply to malic acid also apply to β-hydroxybutyric acid.

On substituting OH in the α-position the effects are:

(1) removal of the propyl but substitution of an ethyl group;

(2) partial neutralisation of the attractive effect of the carboxyl group for H at the α-C atom. Hence we should expect this acid to be a H donator but a much poorer one than β-hydroxybutyric acid. It should be more powerful than malic acid. Experiment confirms this [Quastel and Wooldridge, 1925].

If a hydroxyl be substituted in both α- and β-positions it would be expected that the resulting acid would be a H donator intermediate in activity between α- and β-hydroxybutyric acids and more powerful than tartaric acid. This acid has not yet been investigated.

THE ACTIVATION OF PROPIONIC ACID AND THE EFFECTS OF SUBSTITUENTS.

The activation of propionic acid will be represented thus:

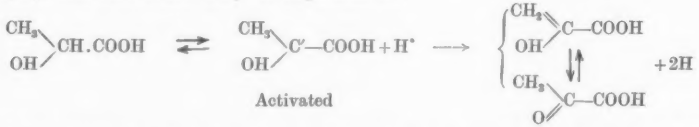
$$\underset{\text{I}}{CH_3.CH_2.COOH} \rightleftarrows \underset{\text{II Normal}}{CH_3.CH'.COOH} + H^{\cdot} \rightleftarrows \underset{\text{III Activated}}{CH_3.\check{C}.COOH} + 2H^{\cdot}$$

The important fact concerning the activation of propionic acid is the repulsive effect by the methyl group on H at the α-C atom (compare succinic acid). Hence the result of activation of propionic acid differs from that of butyric acid where β-oxidation alone can occur. Propionic acid therefore may be expected to be a H donator, its activity being diminished, apparently,

[1] In working with β-hydroxybutyric acid, it is necessary to use a freshly prepared solution. Solutions which have stood for some time, or those which contain bacteria, and exhibit an acetoacetic acid reaction, are not as active as the freshly prepared solutions.

by the non-polar ethyl group. Experiment shows that it denotes H at fairly high concentration [Quastel and Whetham, 1925, 1]. The structure III may, conceivably, produce acrylic acid, but it is much more likely, since the α-C atom attracts (1) negative electricity because of the methyl group, (2) positive electricity because of the carboxyl group, that the elements of water will be added and lactic acid produced (after the 2H have been taken up by a suitable hydrogen acceptor). These views make it clear why butyric acid, in the body, gives rise to acetoacetic acid and propionic acid to lactic acid or sugar.

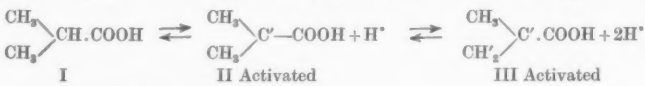
On substituting OH in the α-position we find (1) enhanced repulsion of the α-H and (2) removal of the non-polar ethyl and substitution of the methyl group. Lactic acid, therefore, will be a good donator of H and more powerful than propionic acid. Experiment shows that this is true [Quastel and Whetham, 1925, 1]. Its activation may be represented:



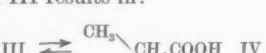
If —COOH be substituted for —CH_3 in lactic acid there should be a very great diminution in hydrogen donating power. Thunberg [1920] has found that tartronic acid is only a very weak donator in presence of muscle.

On substituting —NH_2 in the α-position we expect a similar effect to that following —OH substitution. Owing, however, to the influence of the basic amino-group on the carboxyl it is difficult to estimate the nett effect of amino-substitution. Probably the internal ring formation between amino and carboxyl has a considerable diminishing influence on the activity of the compound. Alanine is a fair donator [Quastel and Whetham, 1925, 2; Thunberg, 1920].

Substitution of a —CH_3 group in the α-position, whilst enhancing the repulsion of a proton from the α-C, considerably increases the non-polar effect of the ethyl group. Hence we expect *iso*butyric acid to be a H donator of about the same order as propionic acid. This is the case [Quastel and Whetham, 1925, 2]. The activation will proceed on the following lines:



Further activation of III results in:



IV will oxidise to the aldehyde, decarboxylate and finally proceed to propionic acid.

Substitution of OH in the α- and β-positions results in:

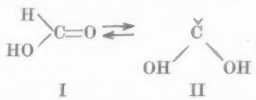
(1) removal of non-polar C_2H_5 and CH_3 groups; (2) removal of the vigorous

repulsive effect on the α-H due to $-CH_3$; (3) partial neutralisation of the attraction for positive electricity at the α-C due to the carboxyl; (4) repulsion of a proton from a β-C to the α-C due to the OH in the β-position.

We expect, then, glyceric acid to be a H donator, not as powerful as lactic acid but more powerful than tartaric acid. This is the case [Quastel and Whetham, 1925, 1].

THE ACTIVATION OF FORMIC ACID AND THE EFFECTS OF SUBSTITUENTS.

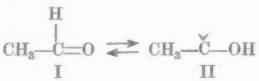
The activation of formic acid is very simply represented:



I II

II is the active form and possesses, because of the hydroxyl groups, a powerful attraction for negative electricity. Hence on activation formic acid should be a powerful H donator. This is the case [Thunberg, 1920; Quastel and Whetham, 1925, 1]. On substituting for OH in I a radicle acting in the same sense, *i.e.* $-NH_2$ or $-CH_3$, the resulting compounds should also be H donators.

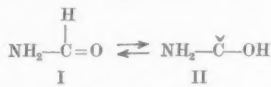
The activation of acetaldehyde is represented thus:



I II

II having a powerful attraction for negative electricity (OH). Owing to the non-polar nature of $-CH_3$ there should be an apparent diminution in the reducing power. Experiment shows acetaldehyde to be a H donator in presence of *B. coli*, its effect being diminished with increase of concentration of the aldehyde (Table IV).

The activation of formamide is represented thus:



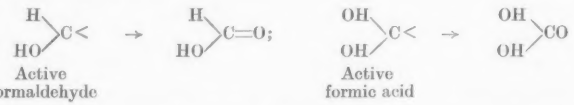
I II

If we accept the formula of formamide as H.C(OH)=NH [Plimmer, 1925] the same activated structure II would be produced. Since the amino-group is polar we expect formamide to be a donator of the same order as formic acid itself. This proves to be the case (Table IV); formamide is active at a concentration of 1/100,000. It may be objected that the activity of formamide may be due to hydrolysis of the latter to formic acid, but from Plimmer's results it seems scarcely likely that the entire activity can be attributed to this cause.

It is interesting to note in this connection the work of Ingold [1924] who has confirmed Nef's view that a hydrogen of acetylene is mobile.

$$CH{\equiv}CH \rightarrow CH_2{-}C{<}$$

This was proved by isolation of the product of oxidation, keten, $CH_2{=}CO$. Ingold suggests that the normal course of oxidation of formaldehyde and formic acid is not that suggested by Bone and Andrew [1905], but one dependent on the active forms of the aldehyde and of formic acid [Baly, Heilbron and Hudson, 1922]. Thus:



These oxidations are uniform with the oxidation of *iso*acetylenes to ketens, of *iso*nitriles to carbimides and of carbon monoxide to carbon dioxide.

Activation of acetic acid and the effects of substituents.

Just as butyric, propionic and formic acids, after activation, exhibit striking differences from one another, so does acetic acid behave in a unique manner. We may represent acetic acid as follows:

$$\underset{\text{I}}{CH_3{-}COOH} \rightleftharpoons \underset{\text{II}}{CH'_2{-}COOH} + H^{\cdot}$$

Now in II there is no particular demand at the α-C for positive electricity as this C already possesses two hydrogen atoms. We will examine what can occur after activation of II. A proton may move from the α-C to an oxygen of the carboxyl and the α-C will be left in a reactive unsaturated state. But in this condition owing now to the demand for positive electricity at the α-C due to the carboxyl, the affinity of the activated structure for hydrogen will be very considerable. It would be unlikely, therefore, that oxidation to glyoxylic acid would occur; it is conceivable that the elements of water would be taken up to produce glycollic acid. This would depend on the relative affinities of the activated structure and, say, methylene blue for hydrogen. But even then it would be doubtful whether —OH would be "accepted" by the structure. Since II is a two carbon group, and, on activation, the movement of a proton can be only in the direction of the carboxyl, it is possible to conceive of a combination of two molecules of II to succinic acid, but this, again, only if the affinity of II for hydrogen is less than that of the methylene blue (or other acceptor in the system). In a three, or more, carbon structure such a synthesis would be unlikely since, on activation, the demand at the α-C for positive electricity would be satisfied by the movement of protons from other parts of the molecule. Hence, on the facts, (1) that acetic acid is a two carbon compound and (2) that two hydrogen atoms are unlikely to be displaced from the α-C owing to the attractive field for positive electricity due to the carboxyl, a synthesis to succinic acid is conceivable. In the case of glycollic acid an oxidation to glyoxylic acid is more likely to occur than one of acetic acid to glycollic acid, but even here the possibility of a synthesis to tartaric acid is not to be ruled out. Experiment shows glycollic and acetic

acids to be H donators of about the same order [Quastel and Whetham, 1925, 1].

The activation of the alcohols and amines.

The reducing power of these substances will depend on the ease of removal of hydrogen from the structures $—CH_2—CH_2—$, $—CH_2—OH$, $—CH_2—NH_2$, and the presence of certain radicles will markedly influence this removal. Methyl alcohol is relatively inert as a donator, demonstrating the stability of the structure $H—CH_2OH$. Ethyl alcohol, as would be expected, is more active and propyl alcohol (which possesses the $—CH_2—CH_2—$ as well as the $—CH_2OH$ structures) still more so. Methylamine and ethylamine are both inactive (in presence of *B. coli*), and indicate the greater difficulty of removal of H from $—CH_2NH_2$ than from $—CH_2OH$. Allylamine is inactive whereas allyl alcohol is a good donator (Table IV).

In the polyhydric alcohols where the non-polar influence of fatty radicles is removed we find glycol to be active and glycerol rather less active [Quastel and Whetham, 1925, 1]. The greater activity of glycol is probably to be attributed to the nearness of approach of the two $—CH_2$ elements. As these become further removed, as in glycerol and erythritol, there is a greater diminution in activity. It is significant that only when the $—CH_2$ elements approach nearest, namely, in the five or six carbon compounds where ring formation is possible, a high degree of activity is obtained. In these latter cases, however, so much complexity is introduced by the stereo-structure of the molecules that it would be fruitless, at the moment, to deal with these in any detail. The same applies to the sugars.

Consideration of energy transference.

Let us consider, again, the structure $—CH_2—CH_2—COOH$ (I) which in presence of, or in contact with, the activating source (muscle tissue or bacteria) produces the system $—\overset{\vee}{C}—CH_2.COOH$ (II) + $2H^{\cdot}$. If the two protons are taken up by a suitable acceptor, II, as has been shown, has three procedures. The energy derived from the reaction

$$\underset{\text{Activated}}{\text{II}} + \quad 2H^{\cdot} + \underset{\text{Acceptor}}{\text{A}} \rightarrow \underset{\text{Normal}}{\text{II}} + AH_2$$

may be entirely dissipated, but it is more likely that, at any rate, part of the energy may go into the structure II and preserve the latter in an activated condition. The molecule then becomes liable to a further reaction.

$$\underset{\text{II}}{\begin{matrix} —C< \\ | \\ COOH—CH_2 \end{matrix}} + \begin{matrix} OH.H \\ OH.H \end{matrix} + A \rightarrow \begin{matrix} —C(OH)_2 \\ | \\ COOH—CH_2 \end{matrix} \rightarrow \underset{\text{III}}{\begin{matrix} —CO \\ | \\ CH_2—COOH \end{matrix}}$$

Thus the energy derived from the first reaction may prevent the accumulation of the normal form of II, *i.e.* the corresponding unsaturated acid (II *a*), and

carry the action on to III. It would appear, then, that I would be oxidised to III, none of II *a* necessarily accumulating. Now if for I we substituted II *a* we should get, after activation, the same product III but it would not necessarily follow that the yield of III from II *a* would be equal to or even be of the same order as that from I. This simply arises from the fact that when II *a* is presented to the activating source it does not contain the extra energy present in II after the first oxidation has occurred.

It is this which, it seems to me, makes intelligible the fact that α-methylphenylacetic acid is fully oxidised in the body whilst phenylacetic acid is not oxidised [Kay and Raper, 1924]. The methyl group in the former, after activation, becomes the reactive point, is oxidised, and the energy, or some part of it, goes into the ring and renders the latter, which is normally fairly stable, susceptible to attack. Phenylacetic acid presents no such "handle" for attack and is moreover rendered inert by removal of the carboxyl by union with glycine.

It is possible also that the energy of an oxidation A $\rightarrow$ AO may be transferred to a molecule of B and render this susceptible to oxidation—an induced oxidation, in effect, ocurring.

Aerobic oxidations.

Hydrogen acceptors fall into two classes—those that require activation by the cell (or those whose oxidising power is increased by the cell), and those whose oxidising power is independent of the cell. To the former belong nitrates, chlorates, fumarates; to the latter, substances of the type of methylene blue. Apart from the possibility that molecular oxygen, unactivated, may act as a H acceptor, there appear to be at least the three following ways in which activation of oxygen can occur:

(1) by iron (Warburg);

(2) by the sulphydryl group. Hopkins [1925], for instance, has shown that cyanide does not prevent the oxidation of linseed oil under the influence of glutathione at p_{H} 7·6.

(3) by substances capable of peroxide formation. Now when the structures —CH=CH—, —CH=O, —CH=N—, become activated to form —$\check{\mathrm{C}}$—$\mathrm{CH_2}$, etc, it seems possible that oxygen may form a temporary link with the activated structure at the unsaturated carbon, thus:

$$\text{—}\check{\mathrm{C}}\text{—X} \rightarrow \text{—}\overset{\mathrm{O—O}}{\overset{\diagup\diagdown}{\mathrm{C}}}\text{—X}$$

this compound having high oxidising power and being in effect "active" oxygen. Hence, the very process which activates the hydrogen donator may result also in activating the oxygen. Possibly the predominating activating influence in a solution containing iron is the iron, but it does not necessarily follow on the above view that if the iron be removed aerobic oxidation will entirely cease.

The specific behaviour of enzymes.

It does not follow from the view of the mechanism of oxidation, which has just been discussed, that there do not exist, in the cells, certain specific enzymes related to oxidation-reduction processes. It appears to me that their presence may be possible, for if a certain structure possesses chemical or physical characteristics closely related to some particular substrate then only the latter may become "accessible," as it were, to the activating power of this structure. Such a structure would be regarded as a specific enzyme. The mechanism of activation, however, would still be explained on the lines indicated in this paper. The cell surface—or indeed an intracellular surface—can, in a sense, be regarded as an assemblage of enzymes, enzymes which are inseparable because they are actually a function of the surface structure.

Perhaps the main aim of this paper has been to indicate not so much that it is unnecessary to postulate the existence of numerous specific enzymes dealing with oxidations and reductions, but that it is possible to regard specificity of behaviour as belonging rather to the molecules themselves than to the enzymes.

Summary.

An attempt has been made, based upon the electrical and polar properties of molecules, to interpret in a consistent way the mechanism of oxidations and reductions *in vivo*. On the basis of the theory which is advanced it is possible to "explain":

1. β-Oxidation.
2. Asymmetric oxidation of the double bond.
3. Difference between the activations of *cis* and *trans* isomers.
4. The α-oxidation of propionic acid.
5. The mode of oxidation of branched chains, and various other oxidation-reduction phenomena *in vivo*.

Predictions have been made and verified.

I wish to express my great indebtedness to Sir F. G. Hopkins, F.R.S., for his ready advice and continued interest in this work, and to Sir J. J. Thomson, O.M., F.R.S., for his kindness in advising me on certain points connected with the electrical theory advanced in this paper.

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XXV. STUDIES ON THE INTERACTION OF AMINO-COMPOUNDS AND CARBOHYDRATES.

I. THE ACTION OF UREA ON GLUCOSE, FRUCTOSE AND MANNOSE.

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From the Department of Physiology, University of St Andrews.

(*Received December 28th, 1925.*)

Many gaps exist in our knowledge of the chemistry of sugar derivatives containing nitrogen, and as, so far, only the more accessible and more stable compounds of this class have been examined from the physiological point of view, very little is known of the behaviour of nitrogenous sugar derivatives in the animal organism.

With the object of furthering our knowledge in this direction, a series of researches was instituted, and glucose ureide was selected as the first compound for investigation. Amongst the reasons which led to the choice of this derivative may be mentioned, firstly, the fact that its preparation had been described by Schoorl [1903], and secondly, that both glucose and urea are important compounds from the physiological point of view. The latter factor acquires a special importance when it is recalled that glucose and urea are present in human blood in equimolecular proportions. Glucose ureide thus promised to be of great biochemical interest, and as the chemistry of the compound appeared fairly complete, the study of its physiological behaviour seemed to depend on the preparation of a sufficient quantity of the compound. Accordingly work was started with this end in view, but it was found that by Schoorl's method considerable difficulty is involved in preparing a large amount of the compound in a state of purity. Alternative methods to effect the condensation were therefore considered, and these led to a study of the action of urea on the simple sugars which forms the subject of the present communication.

Experimental.

The action of urea on glucose in solution in ethyl alcohol.

To an approximately 5 % ethyl alcoholic solution of glucose (1 mol.), which had attained its permanent rotation value, excess of urea (4 mols.) was added, and the resulting solution examined polarimetrically at intervals. Only very slight changes in rotation resulted, but after long standing—for a period of nearly four months—a definite decrease had taken place, for, whilst the initial value for α in a 1 dm. tube was $+ 2{\cdot}44°$, the value then recorded

was only + 1·73°. As no material had separated, the solution was evaporated to dryness *in vacuo* and the residue repeatedly extracted with acetone to remove excess of urea. The product thus obtained was slightly yellowish in colour, of a somewhat pasty consistency, contained only traces of nitrogen and had a marked action on Fehling's solution.

A portion of this material was dissolved in water and the concentration of sugar present then examined polarimetrically and also titrimetrically by Benedict's method. Assuming the sugar to be glucose, its concentration by the former method was 3·2 %, by the latter 4·4 %. Consequently the product could not consist entirely of glucose, and it was then found that the solution under examination gave a marked Selivanoff's reaction [1887], whilst a freshly prepared 4 % solution of glucose gave only a very faint colour with this test.

A repetition of the above experiment using equimolecular proportions of glucose and urea gave similar results, the rotation falling from the initial value of $\alpha = +4{\cdot}23°$ to the constant value of $\alpha = +3{\cdot}44°$ after six months at room temperature.

Analogous results have also been obtained under somewhat modified conditions, an alcoholic solution of glucose and urea in equimolecular proportions being boiled under a reflux condenser for three successive days. With this treatment the rotation of the solution fell from + 1·67° to + 1·41°, and simultaneously a marked Selivanoff's reaction developed.

The action of urea on glucose in aqueous solution.

The action of urea on glucose was then studied in aqueous solution and to economise space the results obtained are summarised in the following tables.

(*a*) *Solvent: distilled water.*

1. Glucose : urea = 1 : 1 = 1 mol. : 3 mols.

	A	B	S	R
At beginning of experiment	+1·89°	+1·906°	–	Neutral
After 6 hours' heating on water bath	0·35	1·916	+++	Alkaline

(A = α observed polarimetrically for $l = 1$; B = α calculated from estimations by Benedict's method; S = Selivanoff's test; R = reaction of the solution.)

2. Glucose : urea = 3 : 1 = 1 mol. : 1 mol.

	A	B	S	R
At beginning of experiment	+1·01°	+1·003°	–	$p_H = 6{\cdot}3$
After 3 hours at 70°	1·02	.	?–	.
„ 2 „ 80°	0·96	.	?+	.
„ 4 „ 80°	0·85	.	+	.
„ 3 „ 85°	0·55	1·003	+++	$p_H > 8{\cdot}0$

(*b*) *Solvent: a phosphate mixture of* $p_H = 6{\cdot}0$ *(reaction of normal urine).*

	Glucose : urea = 1 : 1 (by weight)			Control without urea		
	A	B	S	A	B	S
At beginning of experiment	+0·87°	+0·997°	–	+0·88°	+0·971°	–
After 96 hours at 37°	0·86	.	.	0·88	.	.
„ 8 „ 80–85°	0·33	0·982	++	0·85	0·970	–

(*c*) *Solvent: a phosphate mixture of* $p_H = 7{\cdot}42$ *(reaction of normal blood).*

	Glucose : urea = 1 : 1 (by weight)			Control without urea		
	A	B	S	A	B	S
At beginning of experiment	+0·86°	+0·987°	–	+0·86°	+0·945°	–
After 96 hours at 37°	0·86	.	.	0·86	.	.
„ 2 „ 85°	0·69	.	.	0·60	.	.
„ 8 „ 85°	0·25	0·982	+++	0·52	0·940	+

Further control experiments with glucose in a buffer of $p_H = 7{\cdot}42$ showed that no appreciable change in rotation took place after four months' incubation at 37°, or after heating for three weeks at 50°.

(*d*) *Solvent: a phosphate mixture of* $p_H = 7{\cdot}0$ *(absolute neutrality).*

	Glucose : urea = 3 : 1 (by weight).		
	A	B	S
At beginning of experiment	+0·89°	+0·908°	–
After 2½ hours at 85°	0·83	.	.
„ 4½ „ 85°	0·80	.	.
„ 7½ „ 85°	0·65	0·908	+++

Experiments at body temperature.

The results of the experiments with glucose and urea, reported in the previous section, indicate that a marked change in the nature of the sugar takes place after heating for a few hours at a temperature of about 85°. Accordingly experiments were then carried out to determine whether any change takes place at body temperature. The results of a typical experiment after 3 months at 37° may be summarised as follows.

(1) The p_H increased from an initial value of 6·0 to over 8·0, whilst in a control experiment with the same amount of urea the p_H increased to 7·9, and in a control with glucose no change in reaction was detected.

(2) The observed rotation decreased from + 0·92° to + 0·83°; a control experiment with the same amount of glucose showed no alteration in optical activity.

(3) Selivanoff's and Pinoff's [1905] reactions became slightly positive, the coloration produced being equivalent to that of a 0·05 % solution of fructose, whilst in a control experiment with pure glucose neither of these so-called ketose reactions developed.

Experiments were also conducted to ascertain the effect of Winter and Smith's technique [1923] on a mixture of glucose and urea. The results of these are tabulated as follows:

No. of experiment	Glucose : urea	Initial concentration of sugar		Concentration at end of experiment	
		A, %	B, %	A, %	B, %
1	1 : 1	—	—	1·60	1·86
2	1 : 1	0·183	0·18	0·1714	0·18
3	1 : 1	1·83	1·80	1·77	1·89
4	3 : 1	1·73	1·68	1·73	1·74
5	3 : 1	1·73	1·68	2·13	2·20
6	3 : 1	1·73	1·68	1·92	1·91

(A = % calculated from the polarimetric observation; B = % calculated from the copper reduction value estimated by MacLean's method.)

The results of Exps. 1 to 3 seem to suggest that the presence of urea causes the concentration of the sugar as determined by the polarimeter to be less than that determined by copper reduction methods, but Exps. 4 to 6, on the other hand, indicate that the changes observed are probably due to experimental error. This is consistent with the results of the previous series of experiments which show that definite optical changes can be detected only after prolonged incubation at 37°.

Experiments at 50°.

With the object of confirming the fact that glucose and urea interact at temperatures well below 80°, a series of experiments was conducted at 50°. One typical case may be quoted.

Glucose (9 g.) and urea (3 g.) were dissolved in water and the solution made up to 100 cc. The initial rotation of the solution was then determined and the sugar also estimated by Benedict's volumetric process. Thereafter the solution was incubated at 50°, and the rotation of the solution taken periodically. The original colourless solution soon began to assume a slightly yellowish tint, and after 10 days was yellowish brown. Simultaneously an alkaline reaction developed and with Selivanoff's test the solution then gave positive results, which increased in intensity with the duration of the heating. The optical changes noted were as follows:

Duration of heating in hours	0	240	430	800
α in 1 dm. tube	+4·72°	3·15°	2·10°	1·02°

As after 800 hours' heating no further diminution of optical rotation was detected, the sugar content of the solution was determined by both Benedict's and the Wood-Ost methods. The former gave 8·93 %, and the latter 8·91 % of sugar calculated as glucose: so that both results agreed closely with that calculated from the initial rotation value, namely 8·99 %. Assuming, for the moment, that the final mixture contained only glucose and fructose, use was made of the iodometric method described by Judd [1920] to arrive at the proportions of these two sugars present. From the results obtained, the mixture would appear to consist of 80·74 % glucose and 19·26 % fructose. The ratio of these sugars present was also determined by applying the observations of Nyns [1924] that fructose reduces Ost's reagent at 49° whereas other sugars, such as glucose, do not. By this process, the glucose amounted to 82·27 %, the fructose accounting for 17·73 %. It would then seem that approximately 20 % of the original glucose had been converted into fructose; but the rotation data demand the conversion of at least 25 %.

Decomposition on heating aqueous solutions of urea.

As the changes in optical activity of the glucose solutions containing urea recorded above were always accompanied by the development of an alkaline reaction, it seemed that this was the factor responsible for the fall in rotation. Further, as the alkaline reaction could only be brought about by the forma-

tion of ammonia from the urea, attempts were made to determine whether glucose had any effect upon this decomposition.

Working with a 1 % solution of urea, no ammonia could be detected by Nessler's reagent after 5 days at room temperature (15° to 18°). A similar result was obtained with 1 % urea containing 3 % glucose, and no change in the optical power of the solution was observed. Warming similar solutions at 50° for 20 hours caused the production of 4·73 mg. NH_3 %, and 5·00 mg. NH_3 % respectively. After 44 hours the amounts of ammonia were 9·00 mg. and 9·50 mg. %, whilst after 94 hours 20·07 mg. and 25·00 mg. NH_3 % were produced respectively, and the rotation of the glucose-containing solution had then fallen from the initial value of + 2·70° to + 2·51°. At 85° ammonia was rapidly liberated both in the pure urea solution and in that containing glucose, and, as is evident from the following table, the decomposition proceeded at approximately the same rate.

	1 % urea solution	1 % urea + 3 % glucose solution	
Duration of heating at 85° in hours	NH_3 produced mg. %	NH_3 produced mg. %	α
0	—	—	+2·70°
2	25	20	—
4	35	30	—
6	53	49	0·64°

On the other hand, experiments in which the change in the p_H of the solution was determined indicated that a greater effect resulted on warming at 50° a 1 % solution of urea containing 1 % glucose than a 1 % solution of pure urea, as, in the former the p_H increased from 6·5 to 9·3, and in the latter from 7·1 to 8·5.

The action of dilute ammonia on glucose.

A 2 % solution of glucose containing approximately 60 mg. NH_3 showed no optical change at room temperature, but after 24 hours at 50° the rotation fell from + 0·87° to + 0·29°, and a marked Selivanoff reaction was then obtained.

It was thus evident that ammonia caused a marked lowering of the rotation after heating at 50°, and an experiment at 37° with similar solutions showed after 21 hours a fall from + 0·87° to + 0·71°. No further change took place on longer incubation, the mean of a series of readings taken after 65 hours being + 0·72°.

Similar results were obtained when ammonia was added to an aqueous solution of glucose dissolved in an aqueous phosphate mixture of $p_H = 7·40$.

Although ammonia does affect the rotation of a sugar solution it is much less active in this respect than the equivalent amount of caustic soda, as is shown by the results given below:

	Glucose + ammonia α	Glucose + NaOH α
At beginning of experiment	+0·58°	+0·45°
After 18 hours at 37°	0·47	0·04
„ 40 „ 37	0·41	0·04
„ 18 „ 50	0·40	0·03
„ 40 „ 50	0·16	0·04
„ 120 „ 50	0·16	0·04

Action of urea on other sugars.

Having shown that urea can produce a fall in the rotation of a solution of glucose, it became of interest to determine what its effect would be on other sugars and more especially on the closely related members of the series, namely fructose and mannose.

As was expected, with fructose a marked diminution of the laevo-rotation was observed, whilst with mannose a fall in the dextro-rotation resulted, and a positive reaction with Selivanoff's test was obtained. The results are tabulated below:

(*a*) *Fructose.*

	2 % fructose +2 % urea	2 % fructose
At beginning of experiment	−1·77°	−1·77°
After 1 hour at 85°	1·52	1·75
„ 3 hours at 85°	1·02	1·76
„ 5 „ 85°	0·47	1·77
„ standing 6 weeks at lab. temp.	0·59	1·69*

* This low value is probably due to a black fungus which had developed in the solution.

(*b*) *Mannose.*

	2 % mannose +1 % urea	2 % mannose
At beginning of experiment	+0·48°	+0·47°
After 20 hours at 50°	0·48	0·47
„ 3 weeks at 50°	0·12 (constant)	0·46

Discussion.

It has been shown [Irvine and Gilmour, 1908, 1909; Irvine and Hynd, 1911] that glucose and other reducing carbohydrates condense readily at laboratory temperature with a variety of amino-compounds in ethyl alcoholic solution, provided the latter have the requisite solubilities. As urea is highly soluble in alcohol, it was hoped that this process might prove applicable to the condensation of glucose and urea and thereby furnish a convenient method for the preparation of glucose ureide. On putting this to the test, it was found that the optical activity of an ethyl alcoholic solution of glucose containing excess of urea diminished after long standing to $\frac{5}{6}$ths–$\frac{2}{3}$rds of the initial value.

To account for the decrease in rotation several explanations might be offered. Thus, the change in optical activity observed might be due to the conversion of 25 % of the glucose into the laevo-rotatory glucose ureide, but as glucose ureide is very sparingly soluble in alcohol and as all attempts to isolate from the solution even traces of this compound ended in failure, it seemed that no such condensation had taken place, and accordingly Schoorl's observations are confirmed. The formation of glucose ureide being thus probably excluded, it seemed, from the fact that the mixture now gave a marked Selivanoff reaction, that the alteration in optical activity was due to a change similar to the Lobry de Bruyn transformation [1895, 1, 2; 1896], this having been effected by the interaction of urea in the glucose solution. However, as

during this interaction ammonia would be liberated, the possible production of glucoseimine must not be forgotten, and its formation would furnish another alternative explanation of the fall in dextro-rotation.

The possible importance of this observation in connection with the sugar in urine, blood and other body fluids being recognised, experiments with aqueous solutions were carried out. In these, similar but more marked changes were observed, that is to say no alteration in the copper reducing power of the solution could be detected, but a marked decrease in the rotation of the solution took place, the reaction mixture became alkaline and a strongly positive Selivanoff's reaction developed. These results were obtained when the solvents employed were distilled water or phosphate mixtures of p_H 6·0, 7·42 and 7·0; that is, corresponding respectively to the reactions of normal urine, normal blood and absolute neutrality. Most marked results were recorded when the mixtures were warmed on the water-bath at a temperature of about 80° for a few hours; less marked though quite definite results were obtained when the mixtures were kept in a thermostat at 50° for several days, whilst to obtain definite changes at 37° several months were necessary. It is not surprising, therefore, that, when a mixture of urea and glucose was subjected to a process similar to that employed by Winter and Smith [1923] for obtaining what they considered to be γ-glucose from blood, the slight changes noted could be explained as due to experimental error. Nevertheless, the fact that after a prolonged period definite changes did occur even at body temperature when working with mixtures *in vitro*, suggests that the action of urea and glucose may be of importance *in vivo*, where more than likely the reaction would be catalysed by the presence of a specific enzyme. It might be objected that in the experiment at 37°, the decreased dextro-rotation might be due to the disappearance of a portion of the glucose through the action of micro-organisms. Proof that this was not the case was furnished, not only by no sign of micro-organisms being visible either on naked eye or microscopic examination of the solution, but also by the fact that the final and initial copper reducing powers of the solution were practically identical. Further, later experiments proved that once micro-organisms obtained access to such a mixture of glucose and urea, they developed so rapidly that no further polarimetric examinations of the solutions were possible owing to the turbidity of the latter.

Fructose and mannose have been found to interact with urea in an analogous fashion—the former being more marked in its action than glucose, the latter much less so. As it was thus evident that a similar mechanism was involved in these cases, it seemed futile to duplicate all the experiments, and consequently attention was confined to experiments with glucose with the object of arriving at the actual mechanism of the reaction. This will form the subject of a later paper.

As Schoorl [1903] has observed that fructose, and ketoses generally, do not combine with urea even under the influence of catalysts, the pronounced rise

in rotation observed in the experiment with fructose and urea cannot be due to the formation of fructose ureide. This lends support to the view, already suggested, that ureide formation is not, at any rate, an important factor in accounting for the optical changes observed to occur in alcoholic or aqueous solutions containing glucose and urea. The alternative explanation is accordingly more probable, namely that the ammonia to which the urea gives rise forms glucoseimine or brings about a Lobry de Bruyn conversion.

The latter well-known transformation of glucose into a mixture of glucose, fructose and mannose is known to be brought about by the action of almost any alkali—even a weak alkali such as lead hydroxide. Accordingly it has been suggested by several workers that the glucose of the blood might suffer the change owing to the normal slight alkalinity of this fluid. Though experiments with glucose in a phosphate mixture of $p_H = 7{\cdot}42$ showed a fall in rotation when heated at 85°, no change was detected after 3 weeks at 50° nor after 4 months' incubation at 37°. On the other hand, the presence of an equimolecular proportion of urea, or the addition of 0·06 % ammonia, brings about a definite change in rotation even at body temperature, and a marked fall after 18 hours at 50°.

These experiments, coupled with the fact already mentioned that the normal ratio of glucose to urea in blood approximates 3 to 1, would seem to furnish a clue as to why polarimetric estimations of sugar in nearly all biological fluids almost invariably give lower values than those obtained by volumetric methods. At the same time it must not be forgotten that there are present in nearly all biological fluids substances other than glucose which contribute both to the optical activity and reducing power of deproteinised filtrates. Consequently, as Denis and Hume [1924] have pointed out, the difference between the results obtained by the two methods is only what might be expected. On the other hand, these same authors have obtained differences between the two methods when employing "artificial" blood filtrates where the only interfering substances present were alanine and urea.

Though the partial transformation of glucose into mannose and fructose would account satisfactorily for the optical changes observed and is supported both by the Willstätter and Schudel [1918] and the Wood-Ost [Wood and Berry, 1904] methods for estimating the reducing sugars present, in no single experiment has it been possible to isolate mannose as its phenylhydrazone or to detect fructose by means of methylphenylhydrazine. This was also the experience of Ling and Nanji [1922] with regard to mannose in their work on the action of ammonia on glucose. Accordingly, apart from the fall in rotation and the reduction of Ost's reagent at 50°, proof that fructose is formed is dependent on the production of positive results with Pinoff's and Selivanoff's reactions. The latter of these tests is probably the more reliable, but this test may simply indicate the presence of a reactive sugar and may not be a specific test for ketoses as generally supposed. In any case, colour tests are not sufficient proof of the actual formation of fructose during

the interaction of glucose and urea, and a characteristic derivative of fructose must be isolated before the presence of this sugar can be established. Experiments with this end in view are in progress, and the part played by glucoseimine is also being investigated.

Incidentally a point of clinical importance may be mentioned with regard to the use of Selivanoff's reaction in testing for the presence of fructose in urine. It is generally recognised that urines containing a fair amount of glucose give positive resorcinol tests. In the light of the experiments reported in this paper, the majority of these positive results are probably due, not to the actual excretion of fructose, but to the interaction of urea and glucose. On the other hand, it was found that the addition of urea to a dilute (0·1 %) fructose solution, which gave a distinct Selivanoff reaction, may mask the reaction completely. Consequently this test is of little value for the detection of fructose in solutions containing urea, and its clinical application to urine should, therefore, be discontinued.

Summary.

1. The addition of urea to an alcoholic or aqueous solution of glucose causes a fall in the optical activity of the solution without altering its copper reducing value. The possible importance of this observation is indicated.

2. The optical changes observed are not due to the action of micro-organisms, and ureide formation is probably also excluded.

3. The decrease in optical activity is accompanied by an increase in the p_H of the solution, and also by the development of a positive result with Selivanoff's test.

4. As similar results are obtained with fructose and mannose, the mechanism of the change is regarded as similar to the Lobry de Bruyn transformation, but further experiments are necessary to establish this point definitely, as, in the experiments with urea and glucose, no derivative of either fructose or mannose has so far been isolated, and, further, imine formation is not excluded.

5. It is questioned whether Selivanoff's reaction is specific for ketoses, and it is also pointed out that this test is of little value for the detection of fructose in urine.

6. Experiments are described which suggest that the presence of reducing sugars accelerates the decomposition of urea.

The author takes this opportunity of expressing his indebtedness to Principal Sir James Irvine and Prof. Herring for their kindly interest and helpful criticisms while the work was in progress.

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XXVI. STUDIES ON THE INTERACTION OF AMINO-COMPOUNDS AND CARBOHYDRATES.

II. THE PREPARATION OF GLUCOSE UREIDE.

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A CRYSTALLINE condensation product of glucose and urea was first described by Schoorl [1900, 1, 2; 1903]. So thorough did the last communication appear to be, that, apart from the synthesis of glucose ureide accomplished by Fischer [1914], the condensation of glucose and urea does not seem to have been further investigated.

As indicated in the previous paper [Hynd, 1926], it was found difficult to prepare pure glucose ureide in quantity by Schoorl's method and the author's experience is further corroborated by Hewitt [1913] and personal communications from several workers.

The object of the present paper is to show how, by a modification of the process, glucose ureide can be readily obtained in quantity in a pure condition.

PREPARATION OF GLUCOSE UREIDE BY SCHOORL'S METHOD.

Schoorl's instructions for the preparation of glucose ureide were rigidly followed, and as the method is quoted by Lippmann [1904], it will not be necessary to give details of the process. Working with 90 g. of glucose and the equivalent weight of urea (30 g.) there was obtained, in the first experiment, 26 g. of crude solid, which corresponded to 23·4 % of the theoretical yield. This material was isolated in three different crops of varying purity, weighing 12, 11 and 3 g. respectively. The first crop was readily recrystallised, about 10 g. of pure product being obtained. Attempts to purify the two other crops were quite unsuccessful, so that the total yield of recrystallised material was only 9 % of the theoretical.

In a similar experiment, 50 g. of crude solid material were obtained, corresponding to a 45 % yield; but great difficulty was experienced in purifying this material and the amount of recrystallised glucose ureide was only about 8 % of theory.

Scrutiny of Schoorl's original papers revealed the fact that his yields of pure material were of the same order.

MODIFIED METHOD OF PREPARING GLUCOSE UREIDE.

Formation of an additive compound of urea and glucose ureide.

27 g. glucose were dissolved in about 125 cc. water, 27 g. urea were added, and the resulting solution was filtered. To the clear filtrate 5·6 cc. 25 % sulphuric acid were added and after thorough mixing the rotation of the solution was determined in a 1 dm. tube. The mixture was then placed in a thermostat at 50°, polarimetric observations being made at intervals, some of which are recorded below:

Days	0	1	2	4	7	9
α	+9·05°	8·24°	2·67°	0·69°	0·40°	0·40°

The rotation thus became practically constant after heating at 50° for a week, and at the end of this time the solution was pale greenish yellow in colour, slightly acid to litmus and negative to congo red. As the sulphuric acid had been neutralised during the reaction, the use of barium carbonate was unnecessary, and though the mixture still reduced Fehling's solution vigorously, no attempt was made to eliminate the uncondensed glucose by means of yeast, but the solution, without further treatment, was evaporated under diminished pressure at 37° to a thin syrup. Crystallisation soon commenced and spread rapidly throughout the mass. After standing overnight in the cold, the white, sparkling crystals were drained at the pump, washed with a small amount of 90 % alcohol, and dried in a vacuum desiccator over sulphuric acid. The product thus obtained was practically pure *glucose ureide urea*, and the yield amounted to 31 g. or 73 % of the theoretical.

This preparation has been carried out with amounts of glucose varying from 9 to 180 g., and has always been successful. In the several experiments a total of 450 g. glucose has given 428 g. of the additive compound, which corresponds to a 61 % yield, but in the later experiments, as, for example, that described above, the yield has been somewhat better, so that on an average 66 % of the theoretical amount can be isolated. Other acids, for example phosphoric acid, can be used in place of sulphuric acid to bring about the condensation, but, as they have no special advantages, sulphuric acid is recommended.

Purification and physical properties of glucose ureide urea.

The crude glucose ureide urea was found to melt fairly sharply at about 167° to a colourless liquid, which on cooling resolidified. On raising the temperature above the melting-point, the liquid gradually became yellow, then brown and finally, at about 200°, decomposed with evolution of gas. The solubilities of the additive compound closely parallel those of glucose ureide. Thus it is readily soluble in water (though somewhat less than glucose ureide), very sparingly soluble in either ethyl or methyl alcohol, practically insoluble

in acetone, and quite insoluble in ether, chloroform, and non-hydroxy-solvents generally. Accordingly water was found to be the only suitable crystallising medium, and the use of this solvent has certain drawbacks, owing to the high solubility of glucose ureide urea even in cold water and the fact that on boiling an aqueous solution of the compound, decomposition occurs with evolution of ammonia. Details of the method adopted for the purification are, therefore, appended in the case of an experiment in which 30 g. of crude product were employed.

The solid material was dissolved by pouring over it its own weight (30 cc.) of boiling water. (Complete solution should take place and no filtration should be required, but, if necessary, the solution should first be made more dilute, and, after filtering, concentrated *in vacuo* at a temperature of not more than 40°.) The solution was then allowed to cool slowly, when a crop of crystals separated. These were removed by filtration, washed and dried in a vacuum desiccator. The 17 g. of recrystallised compound thus obtained melted at 170–172° and was evidently quite pure, as the melting-point remained unaltered after several recrystallisations. However, as only a little over 50 % of the material was isolated after each crystallisation from water, the weight of purified product after six recrystallisations was only 3 g., but the accumulated mother liquors on fractional evaporation *in vacuo* at 37° yielded a series of crops of crystals, the weights, melting-points, and specific rotations of which are given below:

	Compound after six recrystallisations	Compound obtained from evaporation *in vacuo*			
		Crop 1	Crop 2	Crop 3	Crop 4
Wt.	3 g.	10·8 g.	6·5 g.	4·7 g.	3·5 g.
M.P.	171–172°	170–172°	170–171°	169–170°	167–169°
$[\alpha]_D$	−18·18°	−17·95°	−18·15°	−17·59°	
	(*c* = 2·5856)	(*c* = 3·636)	(*c* = 7·488)	(*c* = 2·3896)	

As a small fifth crop of less pure material, the melting-point and specific rotation of which were not determined, was obtained in addition to the 28·5 g. of crystalline material detailed above, the process was practically quantitative.

On microscopic examination the crystals were found to consist of stout prisms usually pointed at both ends, somewhat resembling triple phosphates and thus quite distinct in appearance from glucose ureide.

Analysis.

Very little loss of weight (a few mg.) resulted on heating the substance in a steam-oven, or at temperatures from 60 to 100° at 25 to 30 mm. Hg, and as, at the same time, slight browning of the material and lowering of the melting-point (165°) took place, the slight loss recorded was evidently due to decomposition and not to loss of water of crystallisation.

Accordingly, the glucose ureide urea was prepared for analysis by drying in a vacuum desiccator over sulphuric acid.

5 cc. of a 2·5856 % solution of the compound gave 18·16 cc. $N/10$ NH_3 (Kjeldahl).

0·1265 g. gave 0·0758 g. H_2O and 0·1564 g. CO_2.

Found	C = 33·99 %	H = 6·66 %	N = 19·51 %
$(C_7H_{14}O_6N_2) . (CH_4ON_2)$ requires	C = 34·04 %	H = 6·43 %	N = 19·81 %

INTERCONVERSION OF GLUCOSE UREIDE UREA AND GLUCOSE UREIDE.

(*a*) *Conversion of glucose ureide urea into glucose ureide.*

As already indicated, the additive compound of glucose ureide and urea is sufficiently stable to allow of its crystallisation from water provided the temperature is kept about 40°. The compound also remains unchanged after repeated grinding in a mortar with either alcohol or acetone. Thus 6 g. of the compound of melting-point 170°, after four such treatments with about 35 cc. absolute alcohol at a time, melted at 167°, and the united alcoholic extracts and washings on evaporation left only a film. The additive molecule of urea, however, is removed slowly by boiling alcohol.

Glucose ureide urea (70 g.), after 3 days' boiling with several fresh quantities (200 cc.) of absolute ethyl alcohol, was found to yield practically pure glucose ureide (55 g.) melting with decomposition about 200°. This material was recrystallised from water, as described for the additive compound, and the following were obtained:

No. of crop	1	2	3	4
Weight	6·3 g.	6·47 g.	39·9 g.	trace
Melting-point (with decomposition)	208°	207°	207°	205°

making a total of 52·67 g., which is practically a theoretical yield. Crops 1 and 2 crystallised slowly from the solution and consisted of colourless thick rhombic plates, whilst crop 3, which separated quickly from solution, was composed of fine needles. These variations in crystalline appearance do not seem to indicate the existence of different forms of glucose ureide, but depend entirely on the conditions under which crystallisation takes place, for, as shown above, all three crops of crystals had practically the same melting-point. Further, the specific rotation of the plate form was found to be $-23·45°$ ($c = 2·1532$, $l = 2$); that of the needle form $-23·27°$ ($c = 4·5556$, $l = 1$). These values are practically identical and agree with those quoted by Schoorl (− 23·3°) and also by Fischer (− 23·41°). No mutarotation was detected.

(*b*) *Conversion of glucose ureide into glucose ureide urea.*

By crystallising glucose ureide in presence of excess of urea the additive compound was found to be readily formed. Thus, when glucose ureide (12 g.) and urea (6 g.) were dissolved in the minimum quantity of hot water, and the resulting solution allowed to cool, the crystalline material which separated agreed in its properties with those already described for glucose ureide urea.

The reaction between glucose and urea, under the conditions described, no doubt proceeds further than is represented by the 60–70 % of glucose ureide isolated, so that the 30–40 % of syrupy residue contains, in addition to unchanged glucose, some glucose ureide. However, the reaction is not so simple as Schoorl imagined, for evidence has been obtained of the presence in this syrup of some other dextro-rotatory material, and its examination is at present receiving our attention.

SUMMARY.

(1) By the simple modification of using excess of urea in the preparation of glucose ureide, many of the difficulties of Schoorl's method are avoided, and the product is readily isolated as an additive compound (M.P. 170–171°), the conversion of which into glucose ureide (M.P. 207°) is quantitative.

(2) Whereas the yield of pure crystalline glucose ureide obtained by Schoorl's method is only about 10 % of the theoretical, a yield of 66 % can be readily obtained by the method now described.

(3) As the reaction product contains, in addition to the laevo-rotatory glucose ureide and unchanged glucose, some other dextro-rotatory material, the reaction cannot be a simple reversible one, as considered by Schoorl.

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XXVII. STUDIES ON THE BACTERIAL DECOMPOSITION OF TEXTILE FIBRES.

III. THE OCCURRENCE OF HUMUS COMPOUNDS IN DETERIORATED FABRICS AND THE BEARING OF THEIR FORMATION ON THE ORIGIN OF PEAT AND COAL.

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It is now universally conceded that coal owes its origin to the accumulation of vegetable *débris*, which, preliminary to actual carbonisation, underwent a process of decay identical with that occurring today in the formation of peat. This process was described by Fremy [1885] as a "fermentation tourbose," a fermentation process through which the vegetable tissues, or at least the bulk of them, became converted into an amorphous mass of humic substances while isolated tissues escaping decay remained embedded in the amorphous matrix. That Fremy must have regarded micro-organisms as responsible for the decomposition of the plant material is clear, though he gives no information on their nature. Nor does he establish to what extent the chemical units composing the plant tissues partake in the formation.

The decay of vegetable tissues which resulted in the formation of coal subsequent to their conversion into peat is outlined in somewhat greater detail by White and Thiessen [1913], who regard the process as due, at least during its preliminary stage, to the activity of fungi, bacteria playing the chief rôle later. According to White and Thiessen micro-organisms either disintegrate the accumulated plant material completely, in which case amorphous peats are produced, or cease to be active before this stage has been reached. In the latter case xyloid lignites or woody or fibrous peats are formed. The decomposition process is regarded by them as very protracted, in fact they consider that it is still in progress in most of the existing peat bogs even at depths which must have taken one thousand years and more to produce. As regards the effect of the decay on the constituent substances of the plant tissues, White and Thiessen remark that the more labile compounds are removed first, the more resistant later. Finally there remain behind only such parts of the plant material as resins and suberised tissues, which do not usually suffer destruction by micro-organisms. Though cellulose cannot be regarded

as a particularly resistant substance, and, therefore, might be thought to disappear during the process of peat formation, White and Thiessen emphasise the fact that most peats contain a large percentage of cellulose. Its presence as an integral part of peat and coal is accepted by most authorities. Recently, however, Fischer and Schrader [1922] have expressed the view that cellulose, except in insignificant quantities, must undergo complete destruction during the decomposition process which results in the formation of peat. They regard the lignins present as the sole organic constituent of the fuels mentioned, except for the comparatively small quantities of resins and waxes present in the decaying plant tissues.

Fischer and Schrader, and other writers who have dealt with the microbiological aspect of peat and coal formation, base their conclusions on theoretical considerations and advance no convincing experimental evidence in support of their views. This is particularly regrettable since these theories bear unmistakable signs of having been elaborated without sufficient knowledge of the limiting factors of microbiological and particularly of fungal activity.

As the question of the production and origin of humic substances has a bearing on problems on which the writers have been engaged for some time, they have had an opportunity of submitting the microbiological aspect of peat formation to renewed inquiry. The preliminary observations made have been collected in the following notes.

Considering first the *production of peat*, it may be claimed that the decomposition of the peat-yielding plant material takes place under water-logged conditions. From a microbiological point of view this is particularly noteworthy since an important group of micro-organisms, the fungi, thereby becomes eliminated as a serious participant in the microbiological reactions involved. Water-logged conditions are known to prevent the action of fungi on vegetable *débris* to such an extent that immersion in water has been recommended as an infallible means of preserving timber from rot [Snell, 1921]. Of the sparse representation of fungi among the microflora of a typical peat the following experiment bears proof.

The samples of peat were taken from a local bog under precautions sufficiently stringent to prevent subsequent contamination. The actual samples examined, 1 g. in each case, were removed aseptically from the interior of the original samples. Plated out on wort agar which Long and Harsch [1918] recommend for the cultivation of wood-destroying fungi, the samples showed the following numbers of fungi.

Table I.

Sample of peat	No. of fungi found in 1 g. peat
In the surface peat immediately under the vegetation	0
In the peat 1 ft. below the surface	60
„ 2 „ „	30
„ 3 „ „	6
„ 4 „ „	9

In every case the number was so small as to be practically negligible. They show that fungi can have taken no part in the decomposition processes which resulted in the conversion of the vegetation of the bog into this particular peat. The observation agrees entirely with what is known to occur in other, and better investigated decomposition processes or fermentations of water-logged vegetable tissues, *e.g.* the ensilaging of green fodder [Jones and Gibbard, 1923].

It appears, therefore, that the microbiological processes involved in the formation of peat must remain restricted to the activity of bacteria and actinomycetes, groups which contain numerous cellulose-decomposing species and, the former at least, also some which are capable of fermenting lignin [Pringsheim and Fuchs, 1923].

An insight into the extent of the activity of these micro-organisms on water-logged vegetable tissues may be gained from a study of the process of ensilaging, which, as already mentioned, bears striking resemblances to that of peat formation except for the fact that it offers more favourable conditions for microbiological activity than does peat formation.

In broad outline, the action of micro-organisms in the silage heap is restricted to the first three or four weeks after the construction of the heap. The maximum number of bacteria is reached, according to Jones and Gibbard, after 14 days. Subsequently a rapid decrease sets in which results in a complete cessation of microbiological activity 2 to 3 weeks later. By then the silage has frequently acquired the appearance of a young peat, though it still shows the plant tissues composing it in a well-preserved condition. When ripe it is dark brown to blackish in colour and contains an appreciable quantity of humus [Russell, 1908]. In spite of the humification it still contains most of the chemical substances originally present in the raw material, particularly the cellulose and the pentosans on which its feeding value chiefly depends. The following analysis of a typical sample of dark brown sweet silage, taken from the interior of a heap which had been stored on a farm for three years, shows the approximate chemical composition of this fodder.

Table II.

Pentosans	17·54 %
Cellulose	27·36
Nitrogen	1·34
Humic compounds (soluble in 3 % NaOH)	26·00
Ash	20·60
Unaccounted for	7·16

In a series of peat samples it was possible to observe a similar decrease in microbiological activity before the plant tissues had been completely disintegrated. The data recorded below, which illustrate this, were obtained from the analysis of samples of peat taken from a typical peat bog, renowned in the district for its valuable black, well disintegrated peat. The bog is built up of a dense growth of plants, consisting chiefly of low shrubs which

frequently become partly submerged, and reaches a considerable depth, the exact measurement of which could not be established.

Samples of peat, approximately 500 g. each, were taken from this bog from the top immediately below the growing plants and at intervals of a foot down to and inclusive of 4 feet, representing an age ranging from a few years to 40 years approximately. At a depth of 3 and 4 feet the plant material composing the peat was fairly well disintegrated, but it still contained an appreciable proportion of whole tissues mainly in the form of roots. On one occasion a sample of undisintegrated wood with a cellulose content of 34·2 % was detected at a depth of 4 feet. This was found in a place where the upper layers of peat were extremely soft, almost semi-liquid.

The result of the bacteriological analysis (Table III), carried out a few hours after the removal of the samples from the bog, showed that the secondary microflora, consisting of non-cellulose-decomposing bacteria and developing on ordinary laboratory media, had reached its maximum activity in or above the first stratum examined, and that the numbers of this flora, and consequently its activity, were decreasing rapidly with increasing age of the peat.

Table III.

Sample of peat	No. of bacteria present in 1 g. peat: Developing on agar at 37°	Developing on gelatin at 18°	Glucose agar at 37°; anaerobic cultures
Surface peat immediately under the vegetation	19200	200	Nil
Peat 1 ft. below the surface layer	460	104	,,
,, 2 ,, ,,	90	100	,,
,, 3 ,, ,,	1500*	240*	,,
,, 4 ,, ,,	4	Nil	,,

* It is possible that this sample had become infected during collection.

A similar decrease with increasing age was noticeable in the activity of those bacteria which decomposed cellulose. The data given in Table IV illustrate this.

Table IV.

Sample of peat	Filter paper incubated aerobically at 30° after inoculation became attacked after: 7 days	14 days	21 days	35 days	Filter paper incubated anaerobically at 35° after inoculation became attacked after: 7 days	14 days	21 days	35 days
Surface peat immediately below the vegetation	+	+	+	+	−	−	+	+
Peat 1 ft. below the vegetation	−	+	+	+	−	−	−	+
,, 2 ,, ,,	−	−	−	+	−	−	+	+
,, 3 ,, ,,	−	+*	+*	+*	−	−	+*	+*
,, 4 ,, ,,	−	−	−	−	−	−	−	−

* It is possible that this sample had become contaminated during collection.

For the determination of the presence of cellulose-decomposing bacteria, about 1 g. of the fresh peat was inoculated into a medium consisting of filter paper, completely or partly submerged in a solution of peptone and inorganic

salts. This medium, which is very suitable for the cultivation of these bacteria, was recommended for the purpose by McBeth and Scales [1913].

In spite of the favourable conditions thus offered, no cellulose-fermenting bacteria could be developed from the peat taken at a depth of 4 feet, a sample which still contained a percentage of undisintegrated tissues, chiefly roots. Further, the number of cellulose-fermenting bacteria in the strata above must have been decreasing with increasing age of the peat judging by the delay shown by the filter paper in becoming attacked in these cases. That this decrease should have been particularly noticeable in the cultures kept aerobically is not surprising when it is considered that oxygen has little or no opportunity of gaining access to the lower depths of a peat bog where conditions therefore become unfavourable to aerobic types.

Though the observations recorded in Table IV do not justify the conclusion that peat taken from a depth of 4 feet cannot contain micro-organisms in sufficient numbers to indicate microbiological activity, they certainly point to bacterial activity in a peat bog being greatest immediately below the surface of the vegetation, that is, in the youngest layer, and to this activity decreasing as the peat gets older, coming to a standstill while the peat is still young, before all the plant tissues have been disintegrated and before their cellulose has been completely decomposed. Micro-organisms, therefore, cannot be responsible for the elimination of all the cellulose of the peat-forming vegetable tissues. The fact that cellulose-decomposing micro-organisms, fungi or bacteria, are present in, and have taken part in the decay of, peat-forming plants, does not in itself justify Fischer and Schrader's theory of lignin as the only or the chief source of peat and coal. But since cellulose as such eventually does disappear completely in peat, a fact which nobody desires to deny, there must be other agencies than micro-organisms through which this elimination becomes possible. A study of a sample of Egyptian linen cloth dating from the 18th Dynasty has, in the writers' opinion, given a clue to the elucidation of this question. The cloth in question was dark-brownish and very brittle. It had a high alkali solubility and the part of it which went into solution in a hot 3 % sodium hydroxide solution could be precipitated with acid yielding what appeared to be a humic compound. That the deterioration of the cloth had not been caused by micro-organisms was established by the swelling test [Fleming and Thaysen, 1921]. The further examination of the humus-like compound obtained from it yielded results of considerable interest. Following the method used by Eller and his collaborators [1923, 1, 2, 3], a chlorine derivative was prepared from the substance, which on analysis was found to contain 24·17 % of chlorine. It was almost insoluble in ether. In both of these respects it greatly resembled the humus compounds obtainable by the action of acids on carbohydrates. The chlorine derivative of these artificial humus compounds was found by Eller and his collaborators to contain 22·76 % of the halogen, as against 42·3 % in the chlorine derivative of natural humic compounds isolated from a sample of

typical lignite, and 44·5 % in that of an artificial humic compound prepared from quinol. These two highly chlorinated compounds were found by Eller to be readily soluble in ether and are regarded by him as identical substances, definitely distinct from the chlorine derivative of humic compounds prepared from carbohydrates.

That the humic compound collected from the ancient Egyptian fabric had been derived from the cellulose present could not, of course, be definitely proved, though this seemed more than probable since the fabric had been woven from pure linen fibres and had not been dyed.

It was felt that if it were possible to assume that ageing alone might have been responsible for the humification of the cellulose in this fabric and the formation of a compound very similar to, if not identical with, that obtainable from other hexoses, the possibility could not be excluded that similar processes might be responsible for the disappearance of cellulose from peat.

If this had been the case it was to be expected that the humic compounds of peat would consist of a mixture of at least two substances, one representing the cellulose and another the lignin constituents of the original plant tissues.

An attempt was made to determine experimentally whether this was the case. To do so four different peats of varying ages were extracted in the usual way and their chlorine derivatives prepared. These were found to contain the following percentages of the halogen.

Table V.

Humic compound from sample of peat	Percentage of chlorine in its chlorine derivative
Sample I	36·8 %
„ II	36·8
„ III	36·9
„ IV	34·9

As was expected, the percentage of chlorine represented an intermediate stage between that of a typical artificial humus and a typical natural (or lignin) humus.

A treatment of these chlorine derivatives with ether in a Soxhlet extraction apparatus entirely confirmed the assumption of the presence of two different types of humus. By this extraction method it was possible in each case to separate the chlorine derivative into two parts, one soluble in ether, the other insoluble.

The subsequent analysis of the two component parts of these chlorine derivatives gave the following results which confirm the presence of two fractions in the peat humus.

Table VI.

Chlorine derivative of peat humus	Percentage of chlorine in the fraction	
	Insoluble in ether	Soluble in ether
Sample I	21·9 %	40·7 %
„ II	20·6	40·8
„ III	21·6	40·35
„ IV	22·4	41·65

The ether-soluble portion was very similar as regards its chlorine content to that yielded by a typical natural humus; the insoluble portion even more similar to that of a carbohydrate humus than was the case with that isolated from the Egyptian fabric.

SUMMARY.

The experiments discussed in the preceding pages indicate that microbiological activity is insufficient to eliminate all the cellulose present in plant tissues decaying to form peat.

It is shown that the humic compounds which may be obtained from typical peats consist of two different types, one of which yields a chlorine derivative identical with, or very similar to, that of "natural humus" (lignin humic compound), whilst the chlorine compound of the other is closely related to that of the artificial humus compounds obtainable either by the action of inorganic acids on carbohydrates or from cellulose fibres decayed through ageing.

It is suggested that the presence of this latter humus compound in peat may be due to the decomposition of cellulose not eliminated by microbiological activity.

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